National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canada

THE UNIVERSITY OF ALBERTA

MOLECULAR GENETIC CHARACTERIZATION OF BISON

by

ANGELA MARIA BORK (C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

ANIMAL BIOTECHNOLOGY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

SPRING 1990

National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

ISBN 0-315-60230-9

NL-339 (r. 88/04) c

Canada

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR ANGELA MARIA BORK

TITLE OF THESIS MOLECULAR GENETIC CHARACTERIZATION OF BISON

DEGREE FOR WHICH THESIS WAS PRESENTED MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED SPRING 1990

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) Angela Bork

PERMANENT ADDRESS:

Box 64
Chipman, Alberta
T0B 0W0

DATED Nov. 10 1989

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MOLECULAR GENETIC CHARACTERIZATION OF BISON submitted by ANGELA MARIA BORK in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in ANIMAL BIOTECHNOLOGY.

.....
Supervisor

.....

.....

.....

Date.. Nov. 10, 1989.....

DEDICATION

To my parents

Susanne and Guenter Bork

ABSTRACT

One of the oldest and most influential disputes affecting scientists and the public is the classification and associations of Bison. Molecular genetic techniques were employed to evaluate the genetic relationship within and between wood bison (Bison bison athabascae) and plains bison (Bison bison bison) of Elk Island National Park. Genomic DNA samples were screened for restriction fragment length polymorphisms (RFLPs) with cDNA probes for growth hormone (GH), growth hormone releasing factor (GHRF), somatostatin (S), and insulin-like growth factor-I (IGF-I). Of the 28 fragments identified, two revealed RFLPs, both of which were associated with the GHRF locus. The observed frequencies of polymorphic sites did not differ from a Hardy-Weinberg distribution in either population, indicative of random mating populations. Estimates of genetic identity between the populations corresponds closely to the identity found in geographically isolated populations. Analysis of F-statistics, together with the contingency Chi-square analysis for homogeneity, indicated differentiation (P=0.00) between the wood and plains bison. This genetic differentiation is higher than the differentiation reported for other geographically isolated populations; thus, this analysis suggests the bison populations have at least reached geographic isolation in their evolutionary divergence.

The discovery of a 36,000 year old steppe bison (Bison priscus)

has created the potential for applying molecular genetics to resolve the evolutionary history of the bison. Modified DNA extraction techniques were employed to isolate high molecular weight DNA from the prehistoric bison. Hybridization of a bison satellite probe to the prehistoric DNA reveals that a bison-related DNA exists. This identification allows for further comparison of the genetic constitution of this prehistoric bison and modern bison.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my graduate supervisor, Dr. R.K. Salmon for his encouragement, confidence and guidance throughout my thesis program. I would also like to acknowledge Keith as the initiator of my nickname 'Buffalo Bork'.

Dr C. Strobeck and Dr. F.C. Yeh are gratefully acknowledged for their excellent statistical assistance and resource material, both of which helped me gain an appreciation and an understanding of "statistics". I also acknowledge Dr. R.J. Hudson for providing me with valuable information on wildlife management practices and concerns, as well as enlightening me on phylogenetic aspects.

I am grateful to Chuck Blyth for introducing me to my supervisor, Dr. R.K. Salmon. It was Chuck's inexhaustable enthusiasm and creative application which innovated this project, and without his inspiration this work may never have been initiated.

The wardens of Elk Island National Park are thanked for their assistance in blood collection, as well as providing me with important information regarding the bison. Wes Olson is also acknowledged for his artistic aid.

I am especially thankful to Sophie Ogle for her friendship and support. As well, Sophie's experience and excellent knowledge of the lab enabled her to provide me with valuable lab tips which could not be replaced with any manual. I am also thankful to David Glimm for his friendship and great lab suggestions.

I am very grateful to my good friend Olive Dodd, who persevered with me throughout the various stages of my Masters. Her enthusiastic assistance and support from the initial lab stages through to the oral defense were invaluable.

Luc Trenson is thanked for his time and assistance in the lab; as well, Luc's faith and confidence in my work inspired me onward.

I also wish to acknowledge Page Penney, who's precise lab techniques allowed her to share with me the exciting results discovered within the lab. Amber Janzen is also thanked for her lab assistance.

I would like to thank all of my friends: Page Penney, Amber Janzen, Dorothy Payne, Dorothy Irwin and Marina von Keyserlingk for their encouragement, confidence and support which gave me strength to do my best. I am also very grateful to Jack and Shirley Willman and their daughters Kim and Pam for their faith and support in my work.

Last of all, I am grateful to my family, especially Edward, who assisted me in many ways throughout my Masters.

Table of Contents

Chapter		Page
I.	GENERAL INTRODUCTION	1
	1.1 REFERENCES	16
II.	GENETIC RELATIONSHIP OF WOOD AND PLAINS BISON BY RESTRICTION FRAGMENT LENGTH POLYMORPHISMS	19
	2.1 INTRODUCTION	19
	2.2 MATERIALS AND METHODS	22
	2.2.1 SAMPLE COLLECTION	22
	2.2.2 DNA PREPARATION	22
	2.2.3 STATISTICAL ANALYSIS	25
	2.3 RESULTS	28
	2.4 DISCUSSION	31
	2.4.1 INTRA-POPULATION VARIABILITY	31
	2.4.2 INTER-POPULATION VARIABILITY	33
	2.5 REFERENCES	38
III.	IDENTIFICATION OF HIGH MOLECULAR WEIGHT DNA ISOLATED FROM A 36,000 YEAR OLD STEPPE BISON (*B. PRISCUS*)	52
	3.1 INTRODUCTION	52
	3.2 MATERIALS AND METHODS	54
	3.2.1 DNA EXTRACTION AND PURIFICATION	54
	3.2.2 SATELLITE BAND ISOLATION	56
	3.2.3 DOT BLOT AND LABELLING	57
	3.3 RESULTS	58
	3.4 DISCUSSION	60
	3.5 REFERENCES	64

IV. GENERAL DISCUSSION 70

4.1 THEORETICAL CONSIDERATIONS 70

4.2 EVOLUTIONARY IMPLICATIONS 74

4.3 REFERENCES 80

List of Tables

Table		Page
2.1	Restriction fragment lengths associated with growth-regulating loci	45
2.2	Frequency of alleles at polymorphic loci in wood and plains bison and Chi-square test for deviation from Hardy-Weinberg equilibrium	46
2.3	Observed genotypes at Sph I, Eco RV polymorphic sites	47
2.4	Inbreeding coefficient for wood and plains bison at each polymorphic site and estimates of F_{IS}, F_{IT}, and F_{ST} for two polymorphic sites in Bison bison	48

List of Figures

Figure — Page

2.1 Relationship between identified dimorphic restriction fragments and the positions of the variable restriction site. A dimorphic restriction fragment was assumed to reflect the existence of a dimorphic restriction site. In each case where such a situation was assumed to exist, the position of the variable site most proximal to the monomorphic site (labelled X_3) was designated with the subscript '1' (eg. X_1) while the distal position of the variable site was designated with the subscript '2' (eg. X_2) 49

2.2 Autoradiogram of the GHRF-related RFLP's within the wood (W) and plains (P) bison. These polymorphic restriction fragments were revealed when bison DNA was digested with either Eco RV or Sph I. Individual wood and plains bison could be either homozygous or heterozygous for each of the Eco RV and Sph I restriction fragments 50

2.3 Autoradiogram of the European (E) and North American (wood (W) and plains (P)) bison GHRF-related restriction fragments. When European bison DNA was digested with Eco RV the GHRF locus yielded a restriction fragment of 14.4 kb and digestion with Sph I yielded a fragment of 11.1 kb 51

3.1 DNAs of A: human; B: high molecular weight marker; C: Lambda hind marker; D to H: prehistoric bison prior to RNasing. Designations D to H are DNAs isolated from the prehistoric bison from the second to sixth extraction, respectively. The yield of DNA within these samples is greatest within the intermediate extractions (E,F,G) and lowest in the earlier and later (D and H) extractions. The prehistoric DNA is similar in size to the human DNA (>45 kb), and the RNA obtained is in excellent condition with the two major rRNA bands distinctly visible 66

3.2 Column purified prehistoric DNA and plains bison DNA were digested with Eco RI and electrophoresed on a 0.7% agarose gel. A: prehistoric; B: plains bison; C: Lambda hind marker; D: high molecular weight marker. Purification by chromatography had increased digestibility of the prehistoric bison DNA, resulting in fragments ranging from 25 kb to 1 kb; however, the digest pattern differs from that of the plains bison 67

Figure Page

3.3 DNA of plains bison was digested with Pst I and eletrophoresed on a 1% low melt point (LMP) gel to facilitate the isolation of the 700 bp satellite band. A: Pst I restricted plains bison; B: high molecular weight marker; C: Lambda hind marker 68

3.4 Dot blot which reveals the affinity of the bison satellite band to the prehistoric bison DNA and to other control DNAs. This dot blot shows that the prehistoric bison is related to bison and cow but not to human, Drosophila or E. coli 69

I. GENERAL INTRODUCTION

The sun bears down hot upon the rolling plains and, except for the occasional chirp of a grasshopper and the sing song of a meadowlark, all is still, when suddenly over the horizon several hundred, maybe thousand, great hump-backed creatures thunder. In hot pursuit are 50 Plains Indians on horseback, whooping their excitement, arrows drawn on bow, ready for the kill.

To most, the name 'buffalo' creates such a picture; they stir thoughts of the Wild West and convey images of Canada's past. Buffalo have been a part of Canada long before white man came to this vast country and, although relatively few of these animals remain today, they can still be observed and enjoyed within Canada's National Parks. Buffalo, more properly termed as bison (Bison), have become an important Canadian heritage and carry with them a legacy that will not soon be forgotten. Since prehistoric time, the bison has persisted through drastic environmental changes and intense human intervention. Its unique history, both recent and prehistoric, has created several perplexities, resulting in disputes within the scientific realm as well as affecting political decisions.

Fossil remains of bison have been traced as far back as 2.5 million years to the Pliocene (pre-glacial) epoch, where early ancestors were first known to occur in southern and eastern Asia

(McHugh, 1972; McDonald,1981). These primitive animals, although smaller and more slender than present day forms, displayed all the characteristics of the genus Bison. Over the next million years, during an interval known as the Pleistocene or the Ice Age, bison dispersed over much of the northern hemisphere appearing from Europe east through northern Asia into Beringia (McDonald,1981). During the Ice Age, fluctuations in climate created drastic modifications to terrestrial habitat; changes in vegetation type resulted in increased body size in many genera, especially among ungulates (Geist, 1983). It was during this period that bison made their major penetration of North America from Siberia. According to the fossil record of sub-Beringia North America, they first appeared in deposits of Illinoian Age (approximately 300,000 years ago) (McDonald, 1981). As immense glaciers thickened and pushed across the northern hemisphere, so much of the earth's water became secured within these ice masses that sea level dropped drastically and exposed a land bridge between Siberia and America. It was this land bridge, the Bering Land Bridge, that made possible the migration of the larger-horned steppe bison (Bison priscus) into Alaska.

Over the next 300,000 years, environmental changes in North America resulted in the evolution of other distinctly recognizable varieties of bison. Continued development within these early ancestors eventually gave rise to modern day bison, the wood bison (Bison bison athabascae) and the plains bison (Bison bison bison). The Spanish explorer, Hernando Cortez, was the first white man to

have seen bison in the New World. Cortez witnessed the bison in 1521 in a menagerie retained by the Montezuma near Mexico City (Dary, 1974). This Mexican bull, was considered by Cortez the greatest "Rarity" in the animal collection, exhibiting a fierceness and agility no less than that of a fighting bull. Weighing half a ton more than his nearest rivals, the moose and the big brown (Kodiak) bear, the bison is the largest land mammal in the New World (McHugh, 1972). Immense size and unique body conformation contribute to his powerful and majestic stature. The forequarters of the body is colossal and broad; the hind part, in proportion, is small and weak bestowing a strong yet agile appearance. The massive head is carried low, the short, solid neck suddenly extends to a very high wither which then gradually slopes down to a small croup. The immense forepart is further accentuated with long hair, which covers the head, neck, shoulders and thighs. This cape extends up to the shoulder blade and is strictly defined from the hind part where the hair is short and smooth.

Simultaneous to the evolution of bison in North America, was the development of a close relative in the Old World, the European wisent (Bison bonasus). Although the wisent closely resembles the American bison in skeleton and general appearance, it is more streamlined, with longer legs, a longer and lighter body and a less pronounced hump (Geist and Karsten, 1977). While both bison forms can definitely be traced to a common ancestry, the degree of relatedness between the two species (the European and the North American) remains

questionable. A reservoir of an ancient bison may have produced migrants that dispersed into Europe and across the land bridge into America, resulting in parallel lines of evolution (McHugh, 1972). On the other hand, the European wisent itself may have crossed the land bridge during its last connection (approximately 10,000 years ago) and given rise to the North American species.

In addition to the problem dealing with the evolutionary relationship, remains the dispute as to which of the three bison types (the wisent, wood bison or plains bison) is the most evolved. It is generally believed that bison at the start of the Ice Age were large and over time they developed smaller horns and bones. Paleontologists, in the past, have traced early bison through the Ice Age basing their findings on the shape and size of the horns. However, the conclusions drawn from this type of analysis have been rendered unreliable, since variation in horn size exists within species as well as between species. Furthermore, horns will increase in size with age and can change according to diet, physical accidents and general health (McHugh, 1972).

Relying primarily on morphologies and time depths, the majority of evolutionary pathways proposed for the bison place the North American species as the most evolved. However, at present two types of bison reside within North America further complicating the issue. Since the wood and plains bison appear simultaneously in the fossil record, it is difficult to determine which subspecies is most recent. Moreover, the similarity between the subspecies has created

uncertainty towards their distinction.

Historically, the range of the wood bison (Bison bison athabascae) extended from Great Slave Lake, south through the Rocky Mountains to Mexico and west to the Pacific states. East of the Rockies, stretched the range of the plains bison (Bison bison bison), across the continent to the Atlantic Ocean. The homeland of the plains bison was the Great Plains, extending from the tip of Texas up to southern Alberta, Saskatchewan, and Manitoba (McHugh, 1972). Size, as well as color and texture of the coat, are useful parameters in distinguishing the two subspecies. The average male wood bison stands 182 cm at the shoulder and weighs up to 1300 kg; whereas the average male plains bison is 165 cm at the shoulder and weighs up to 1000 kg. The plains bison possesses more hair in the forequarters owing to a larger head, more pronounced cape and distinct skirt on the forelegs. In color, the wood bison is darker, and his hair is more dense and silky.

Although the wood and plains bison differ in physical parameters and occupy variant habitats, their classification as separate subspecies has remained a dispute for nearly 200 years. Sir William Butler in 1810 commented:

> It is still a matter of dispute whether the wood buffalo is the same species as his namesake of the southern plains; He is larger, darker and wilder, he seeks in preference the thickest woods. Whether he be of the plain race or not, one thing is certain - his habits vary much from his southern cousin (Roe, 1970).

To account for the variation in habit and appearance, it has been

suggested that these differences may be attributed to environmental influences. Warburton Pike (1889) wrote:

> ...most people are of the opinion that they (the wood buffalo) are a distinct race from the old prairie buffalo so numerous in bygone days; but I am inclined to think that the very slight difference in appearance is easily accounted for by climatic influences, variety of food and the better shelter of the woods (Roe, 1970).

Whether the differences are a result of environment or genetics, one hundred years ago the wood and plains bison may indeed have been two very separate and distinguishable subspecies. However, recent history involving the extermination of the 'teeming myriads' of bison and the mismanagement of the survivors, has created further skepticism.

In 1871, bison wandered across the land in astonishing multitudes. Of all the total bison estimates, the most accurate was probably that calculation of Ernest Thompson Seton (Dary, 1974). Before white man's arrival, Seton figured there were 75 million bison in North America, including those that inhabited the prairies, plains and forested areas. In the closing years of the 1800's, when white man came west, the millions of bison began to disappear from the grassland. J. Frank Dobie observed:

> Nearly all men were 'wrathy to kill' buffaloes above all else. Mountain men, emigrants crossing the plains, Santa Fe traders, railroad builders, Indian fighters, settlers on the edge of the plains, European sportsmen all slaughtered... (Dary, 1974).

The bison were not only eliminated for sport, but also to overthrow

the Indian since he blocked the way West for the white man. The bison herds roaming the western prairies made the tribes self sufficient and, as long as there were bison, the Indians maintained their strength, courage and freedom. Without the bison, the Plains Indians were conquered and land taken by ranchers and settlers alike (Dary, 1974).

Even without the Indians, these herds of bison were an 'insufferable nuisance'. They ace the grass that was wanted for rancher's cattle and trampled the land the settlers wanted to till for crops (Dary, 1974).

By 1880, the plains bison was virtually exterminated in Canada. This eradication prompted the Canadian government to conserve the last bison, and in 1893 the Federal Game Legislation was established specifically to protect the bison.

Concurrent with the slaughter, a handful of concerned men hung up their rifles to preserve the bison. One of the men responsible for saving the plains bison was a Pend d'Oreille Indian named Walking Coyote. In 1872, Walking Coyote had initially collected four calves (two males, two females) to use as a peace offering. As it turned out, he kept the calves and by 1884 his small herd had grown to thirteen bison. The bison became difficult to control and reluctantly Walking Coyote put them up for sale. Two ranchers, Charles A. Allard and Michel Pablo, bought ten of the animals from Walking Coyote and over the next ten years the herd prospered. At this time, new blood was introduced into the herd, when an additional

26 bison were bought from C.J. "Buffalo" Jones. In 1895, upon the death of Allard, the bison (approximately 300) were equally divided between Allard's estate and Pablo. Allard's half of the herd was sold to various ranchers in the United States and fifteen went to Yellowstone National Park. In 1906, the Flathead reservation (in Montana) used by Pablo's bison was opened to homesteaders, forcing him to sell his herd. Pablo first offered his bison to the United States government and when congress refused him, Pablo approached the Canadian government. In 1906, the Canadian government became owners of over 700 plains bison.

It took over six years to transport 716 bison by railroad from Montana to Alberta. The first two bison shipments were released into Elk Island National Park (EINP) as the original refuge for the bison, Wainwright Park, was still under construction. Once Wainwright Park was completed, the bison originally sent to EINP were rounded up and transported to Wainwright. Despite efforts to move them all, approximately 85 hid themselves in the rougher parts of the country and could not be removed. These bison have become the nucleus of the herd that exists in EINP today.

At the same time, the wood bison were following a similar fate as the plains bison; in 1893, less than 300 wood bison could be located in Canada. Since these were the last wood bison left in the world, they were placed under the protection of the Royal Canadian Mounted Police so they could prosper and multiply without human interference. By 1922, the wood bison's protected range, which now

supported 1500 animals, became Wood Buffalo National Park (WBNP).

Within Wainwright Park, the plains bison did extremely well and by 1925 an astounding 10,000 bison were wandering about the over-grazed prairies (Ogilivie, 1979). Since this number of animals exceeded the carrying capacity of the park, a decision had to be made with regards to the management of the plains bison. Proposals to reduce the numbers through wholesale slaughter met with public outcry. The only alternative was to ship the bison to another park. In the north lay WBNP, all 10,000 square miles of it inhabited by a mere 1500 wood bison. Although naturalists and zoologists argued that the incoming plains bison would crossbreed with the wood bison, it was assumed by government decision makers that the ranges between the wood and plains bison would be separated by a tract of rough and impassable country. In 1925, the Department of the Interior warranted the release of 6,673 plains bison into WBNP.

As had been predicted by scientists, the wood and plains bison began interbreeding and before long the once full-blood herd of wood bison became an "interbred mass of hybrids" (Ogilivie, 1979). Despite continued crossbreeding between the two bison types, biologists suspected that a remnant of pure wood bison may have remained untouched within the park. At last, during an aerial survey in 1957, N.S. Novakowski of the Canadian Wildlife Service discovered such an isolated population of bison in the northwest corner of the park. These bison, approximately 200, were separated from the hybrids by a strip of inhospitable timber and bog (McHugh, 1972). In

order to verify the subspecific identity, five specimen were collected and examined at the National Museum of Canada where they were identified as true wood bison. Between 1963 and 1965, eighteen of the recovered bison were transferred to the Mackenzie Bison Sanctuary (MBS) in the Northwest Territories and twenty-two to the southern part of EINP, where they are managed separately from the plains bison.

The introduction of plains bison into WBNP has created new concerns toward the subspecific classification of the North American bison. The measurements performed on the five specimen collected typify wood bison; however, these recovered bison may have had a low amount of plains contamination and exhibit characteristics resembling wood bison. The purity of the existing wood populations in the MBS and EINP remains unknown. If the present wood bison have been contaminated with plains bison, then it must be questioned whether they are 'meritorious' of a separate subspecies classification.

Since physical parameters can vary according to diet, health and environmental influences, the most assured method to proclaim the bison as distinct is to identify variation at the molecular level. In an attempt to distinguish wood and plains bison, several genetic and biochemical studies involving blood typing (Zamora, 1983; Peden and Kraay, 1979), karyotyping (Ying and Peden, 1977), as well as analysis of red cell carbonic anhydrase (Peden and Kraay, 1979), and mitochondrial DNA analysis (Cronin, 1986) have been performed. Investigation into these various parameters revealed no significant

variation between the wood and plains bison. Since the properties used to delimit a species or subspecies can vary with taxonomists, the most logical and direct approach is to analyze the information that constitutes an individual organism, that is its genetic make-up. Ralston III (1985) defines a species as "a coherent ongoing form of life expressed in organisms, encoded in gene flow, and shaped by the environment". The genetic code of an organism is less likely to vary with external pressures than any of the other parameters selected for taxonomic differentiation, and is therefore the preferred choice for investigation.

A novel genetic approach becoming popular for determining relationships within and between wildlife populations, is the analysis of genomic DNA by restriction fragment length polymorphisms (RFLPs). RFLPs are genetic markers which identify variation (polymorphism) within specific DNA fragments. By the action of restriction endonucleases, nuclear DNA is cleaved into fragments, and polymorphisms which alter the length of the DNA fragments result in RFLPs. Theoretically, individuals which comprise a species/subspecies will possess unique fragments that are not present within other groups of organisms. Analysis for RFLPs within wood and plains bison could provide valuable information with respect to the structure of bison populations, as well as to the relationship between the two bison forms.

In theory, functional characteristics in relation to gene flow can be used to define population boundaries, which are beneficial for

pooling populations into meaningful categories (Smith et al., 1975). Subjecting individuals from various localities to RFLP analysis would give an indication of migration rate (Slatkin, 1989), and of the genetic differences within and between populations (Nei, 1972). A unique DNA fragment pattern specific for one group of individuals would assist in the differentiation of closely related species. Also, significant differences in allele frequencies across geographic areas, can be used to define functional populations which may or may not differ in other characteristics (Smith et al., 1975). The identification of polymorphic DNA fragments within and between groups of humans (Lubahn et al., 1987; Kohenen-Corish et al., 1986), mice (Figueroa et al., 1988; Wakeland et al., 1986) and deer species (Lima de Faria et al., 1986) has been successfully performed by way of RFLP analysis, and has allowed scientists to determine inter- and intra-population relatedness. Establishing the association between such populations or groups yields important evolutionary implications which could assist in the development of phylogenetic patterns.

With the advent of RFLP analysis for identifying genetic differences between individuals and populations, wildlife managers now have a powerful tool to aid in law enforcement efforts in protecting endangered species, and to assist in the selection of animals for restocking purposes. The illegal harvest of endangered or threatened species by poachers poses a primary concern because of inadequate wildlife identification techniques. With the positive identification of a poached species, law enforcement officers have

evidence to subject violators to their rightful punishment. As well, the lack of information on population structure, and on the amount of subdivision within a group of organisms, has had serious impacts within restocking programs (Ryman and Stahl, 1981). The introduction of "wrong" stock may have disastrous effects as occurred with the mixing of wood and plains bison in Wood Buffalo National Park.

In the maintenance of a viable population, the assessment on the availability of genetic variation is essential, and also has important evolutionary consequences (Soule, 1986). When a population is confronted with changing environmental conditions, ideally the population's gene bank is maintained at a high level of diversity to withstand extinction (Smith et al., 1975). With no convenient method to assess the degree of genetic variation or to calculate the amount of inbreeding, much money has been spent in an effort to determine the causes for fitness deterioration within wildlife populations. In one such case, deer having small racks were thought to be inbred; however, the primary problem was indeed related to overpopulation or habitat quality (Smith et al., 1975). Over time, the level of genetic variability could be monitored by RFLP analysis which would provide a sensitive indicator for evaluating the effectiveness of management programs. An estimate of the genetic diversity within individuals and populations is useful for maximizing variability in newly introduced populations, and within groups of endangered species. The ability to determine lineages within a group of

organisms would allow managers to arrange matings between diverse individuals. In one particular study, RFLP analysis was used to determine whether parental mixing occurred within the monogamous species, the snow goose (Anser caerulescens caerulescens) (Quinn et al., 1987). To understand the mating strategy of a species is particularly beneficial within managed breeding programs to ensure maximum gene diversity for future generations. This is pertinent to the management of the bison, since the populations that exist today originate from a very small number of founders.

Additional genetic research may examine possible genetic relationships with desirable phenotypic characteristics (cause and effect relationships) to assist in improving wildlife management techniques (Smith et al.,1975). Accurate estimates on the importance of genetic and environmental components for variation in characters such as growth rate, survival and disease susceptibility are important factors influencing the well-being of a species. Looking into the future, new recombinant DNA techniques will make the transfer of genes among individuals possible, which could be advantageous to a species existence, especially within this rapidly changing environment.

The association between groups of North American bison, and the relationship of New and Old World bison has mystified scientists and politicians for many years. Since 1893, Canada has expended considerable time, effort and money in managing the wood and plains

bison separately, under the assumption that the two North American forms are distinct. The application of RFLP analysis to the bison predicament may assist in the characterization of populations as well as aid in the differentiation of the two North American bison types. Information pertaining to the amount of genetic diversity and to the mating strategy of bison would aid in management decisions to improve the viability of existing populations. This type of genetic analysis can also be applied to the evolutionary dispute; an estimate on the degree of relatedness between North American and European bison may contribute to the resolution of phylogenetic patterns.

Bison have been a part of Canada long before white man's arrival, and the Wild West is remembered and relived because of this immense, powerful animal. Since these great hump-backed creatures are an important component of Canada's heritage, there is an obligation to preserve and maintain healthy bison populations for future Canadians to enjoy.

1.1 REFERENCES

Cronin, M.A. 1986. Genetic relationships between white-tailed deer, mule deer and other large mammals inferred from mitochondrial DNA analysis. M. Sc. Thesis. Montana State University, Bozeman, Montana.

Dary, D.A. 1974. The buffalo book. The Swallow Press Inc., Chicago.

Figueroa, F., Neufeld, E., Ritte, U., Klein, J. 1988. t-specific DNA polymorphisms among wild mice from Israel and Spain. Genetics 119:157-160.

Geist, V., Karsten, P. 1977. The wood bison (Bison bison athabascae Rhoads) in relation to hypotheses on the origin of the American bison (Bison bison Linnaeus). Z. Saugetierk 42:119-127.

Geist, V. 1983. On the evolution of Ice Age mammals and its significance to an understanding of speciations. Walton Distinguished Lecture Series, Mountain Lake Biological Station 30:109-133.

Kohenen-Corish, M.R.J., Serjeantson, S.W. 1986. RFLP analysis of HLA-DR and DQ genes and their linkage relationships in the Pacific. Am. J. Hum. Genet. 39:751-762.

Lima de Faria, A., Arnason, U., Widegren, B., Essen-Moller, J., Isaksson, M., Olsson, E., Jaworska, H. 1984. Conservation of repetitive DNA sequences in deer species studied by southern blot transfer. J. Mol. Evol. 20:17-24.

Lubahn, D.B., Lord, S.T., Bosco, J., Kirshtein, J., Jeffries, O.J.,

Parker, N., Lertzow, C., Silverman, L.M., Graham, J.B. 1987. Population genetics of coagulant factor IX: frequencies of two DNA polymorphisms in five ethnic groups. Am. J. Hum. Genet. 40:527-536.

McDonald, J.N. 1981. North American bison, their classification and evolution. University of California Press, Berkeley, Los Angeles, London.

McHugh, T. 1972. The time of the buffalo. Alfred A. Knopf Inc., New York.

Nei, M. 1972. Genetic distance between populations. Amer. Nat. 106:283-292.

Ogilivie, S.C. 1979. The park buffalo. Calgary-Banff Chapter. National and Provincial Park Association, Canada.

Peden, D.G., Kraay, G.J. 1979. Comparison of blood characteristics in plains bison, wood bison, and their hybrids. Can. J. Zool. 57:1778-1784.

Quinn, T.W., Quinn, J.S., Cooke, F., White, B.N. 1987. DNA marker analysis detects multiple maternity and paternity in single broods of the lesser snow goose. Nature 326:392-394.

Ralston III, H. 1985. Duties to endangered species. Bioscience 35:718-726.

Ryman, N., Stahl, G. 1981. Genetic perspectives of the identification and conservation of Scandinavian stocks of fish. Can. J. Fish. Aquat. Sci. 38:1562-1575.

Roe, F.G. 1970. The North American buffalo. University of Toronto

Press, Toronto and Buffalo.

Slatkin, M. 1989. Detecting small amounts of gene flow from phylogenies of alleles. Genetics 121:609-612.

Smith, M.H., Hillestad, H.O., Manlove, M.N., Marchinton, R.L. 1975. Use of population genetics data for the management of fish and wildlife populations. Forty-First North American Wildlife Conference.

Soule, M.E. 1986. What do genetics and ecology tell us about the design of nature reserves? Biol Conserv. 35:19-40.

Wakeland, E.K., McIndoe, R.A., McConnell, T.J. 1986. Genetic diversity of class II genes in wild mice: definition of five evolutionary groups by RFLP analysis of A_β and A_α. J. Immunol. 27:255-260.

Ying, K.L., Peden, D.G. 1977. Chromosomal homology of wood bison and plains bison. Can. J. Zool. 55:1759-1762.

Zamora, L.E. 1983. An analysis of bison erythrocyte antigens and blood proteins. M. Sc. Thesis. Texas A & M University.

II. GENETIC RELATIONSHIP OF WOOD AND PLAINS BISON BASED ON RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

2.1 INTRODUCTION

The existence of a distinct form of bison in northern Canada was recognized over 100 years ago and eventually culminated in the designation of this northern form as the subspecies Bison bison athabascae, commonly known as the wood bison (van Zyll de Jong, 1986). However, the lack of an unambiguous concept of a subspecies has resulted in a persistent controversy concerning the validity of this subspecific designation as distinct from the plains bison (Bison bison bison) (Peden and Kraay, 1979; Mcdonald, 1981; van Zyll de Jong, 1986). Wood bison are usually distinguished from plains bison by their larger body parameters, larger horn cores, dark woolly pelage and a relatively small amount of hair on their upper forelegs and beard (Geist and Karsten, 1977; Reynolds et al., 1982). Employing multivariate morphometrics to analyze body size parameters, van Zyll de Jong (1986) considered that the phenotypic discontinuity in body size parameters fully justified the subspecific distinctions However, the conclusion of van Zyll de Jong (1986) is inconsistent with the previous observations of Peden and Kraay (1979) who found that the blood types and carbonic anhydrase polymorphisms in wood, plains and wood x plains hybrid bison were similar. On the

basis of their study, Peden and Kraay (1979) argued that the classification of wood and plains bison as separate subspecies could be questioned. Other comparisons of the blood types and karyotypes of wood and plains bison (Ying and Peden, 1977; Zamora, 1983) have been no more successful in clarifying the taxonomic relationships between the two bison types.

Several recent studies have revealed the potential of restriction endonuclease analysis of mitochondrial DNA to clarify the genetic relationships and evolutionary histories of various groups of organisms (eg. Avise et al., 1979b; Ferris et al., 1983a; Ferris et al., 1983b; Powell, 1983; Wright et al., 1983; Spolsky and Uzzell, 1986). However, preliminary work with wood and plains bison mitochondrial DNA has been inconclusive as additional comparisons between the subspecies are required to establish criteria for classification (Cronin, 1986).

Researchers in the past have opted for molecular parameters that had no obvious relationship to the characteristic phenotypic differences between the two bison types, and this may explain why previous studies have been unsuccessful. There is no clear reason why wood and plains bison would differ in molecular parameters that have had no obvious selection pressures to cause their divergence. Studies which examine the structure and/or expression of genes, with an established involvement in the manifestation of the divergent phenotypic characters, may therefore be more effective in clarifying the genetic relationship between wood and plains bison.

Theoretically, if the divergent body size parameters of wood and plains bison are genetically (rather than environmentally) determined, variation must exist at growth-regulating genes within the two bison types. In a study of lines of mice with divergent growth patterns, Salmon et al. (1988) employed restriction endonuclease analysis of the growth hormone gene locus to identify genetic variation which was strongly associated with body size. Therefore, the study reported herein employed restriction endonuclease analysis of four genes (growth hormone, growth hormone releasing factor, somatostatin, insulin-like growth factor-I), known to regulate postnatal mammalian growth (Raisz and Kream, 1981; Froesch et al., 1985; Zapf and Froesch, 1986; Gorbman et al., 1983), in order to explore the genetic relationships between the two North American bison types.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

Approximately 10 ml of blood was drawn from the tail vein of 40 plains bison and 40 wood bison randomly selected from the two bison populations maintained in Elk Island National Park (EINP). The blood was collected in EDTA (ethylene-diaminetetraacetate) vacutainers (Beckton Dickinson and Company, Rutherford, NJ) and immediately stored between 0°C and 4°C (wet ice or refrigerator) until DNA extraction.

Four European bison (wisent) samples were obtained for comparison to polymorphic loci discovered between the two North American bison subspecies. Three wisent DNA samples, which originated in Poland, were generously provided by Dr. Fellous (Institut Pasteur, France). The fourth wisent blood sample was provided by C.G. Penny (San Diego Zoo, California).

2.2.2 DNA preparation

To facilitate the extraction of DNA, white blood cells were isolated and subsequently lysed. To isolate white blood cells, 5 volumes of a 0.155 M NH_4Cl/0.17 M Tris solution (prewarmed to 37°C) was added to 1 volume of whole blood. Following a 5 min incubation at 37°C, the solution was centrifuged at 2000 rpm for 10 min. After aspiration of the supernatant, the pellet was resuspended in 10 ml of a 0.85% NaCl solution and centrifuged at 2000 rpm for 10

min. This sequence of aspiration, resuspension in 0.85% NaCl, centrifugation and aspiration was then repeated. The white blood cells in the final pellet were lysed by resuspending this pellet in 2 ml of 100 mM Tris (pH 8.0)/1 mM EDTA and then immediately injecting 2 ml of 100 mM Tris (ph 8.0)/40 mM EDTA/1.2% SDS (sodium dodecyl sulfate).

For extraction of DNA, an equal volume of TE (10 mM Tris Cl (pH 8.0)/1 mM EDTA (pH 8.0))-saturated phenol was added to the solution of lysed cells and mixed for 10 min. The resulting emulsion was then centrifuged at 5000 rpm for 5 min. After centrifugation, the upper aqueous phase was re-extracted with an equal volume of TE-saturated phenol. This second phenol extraction was followed by an extraction with an equal volume of 1:1 phenol/chloroform and, subsequently, with an equal volume of 24:1 chloroform/isoamyl alcohol. The final aqueous phase was then dialysed against 3 changes of TE over a 24 hour period. Following dialysis, DNA was ethanol precipitated and resuspended in TE.

A total of 10 commercially obtained restriction endonucleases (Bethesda Research Laboratories, Burlington ON; Pharmacia, Dorval QB) were used to digest the bison DNAs. Addition of the restriction enzyme was carried out over 6 h and total digestion time ranged between 18 and 22 h. The digested DNAs were electrophoresed on 0.7% agarose gels in a 0.04 M Tris-acetate/0.001 M EDTA (Maniatis et al., 1982) buffer at 30 v for 20 to 24 h. Ethidium bromide (0.3 ug/ml) was added to the gel to allow visualization of DNA. Each gel

included DNA markers of known fragment sizes: bacteriophage lambda cleaved with Hind III; pBr322 cleaved with Sau3A I; as well as high molecular weight (HMW) (BRL) marker. The size of the marker fragments ranged from 48 kb to 0.36 kb to allow for an estimation of bison fragment size. The restricted DNAs were transferred onto a nylon membrane (GeneScreen Plus, New England Nuclear Research Products, Boston MA). Treatment of the DNA before and after transfer to the membrane followed the conditions recommended by the supplier. Method of transfer followed the protocol outlined in Maniatis et al. (1982). Transfer was carried out for 38 to 48 h.

The four cDNAs (complementary DNAs) used for hybridization analysis were: bovine growth hormone (Gordon et al., 1983); human somatostatin (Shen et al., 1982); human insulin-like growth factor-I (Rotwein, 1986); and rat growth hormone releasing factor (Mayo et al., 1985). Plasmids containing the above cDNA were transformed into appropriate host bacterial strains, isolated, and purified following the procedures outlined in Maniatis et al. (1982). Inserts were cleaved from plasmids by restriction enzyme digestion and separated from vector DNA on LMP agarose (BRL) gels. Probes were radiolabelled with [^{32}P]dCTP (ICN) by the random primer method (Feinberg & Vogelstein, 1983; 1984). The labelled probe fragments were separated from the unincorporated radionucleotides by spun column chromatography (Maniatis, 1982). Prehybridization, hybridization and washing of the membrane followed the formamide procedure recommended by the supplier. Membranes were autoradiographed at -70^{o}C using

two sheets of Kodak GBX-2 film (Eastman Kodak Co., Rochester, NY) and Lightning Plus intensifying screens (Dupont). Films were exposed for 3 to 14 days depending on probe activity.

2.2.3 Statistical analysis

The fragments, identified via autoradiography, were considered in terms of restriction site locality. While more complex sequence alterations could undoubtedly occur, the assumption was made that a dimorphic restriction fragment reflected the existence of a dimorphic restriction site (Figure 1). Where such a situation was assumed to exist, the position of the variable site most proximal to the monomorphic site (labelled X_3 in Figure 1) was designated with the subscript '1' (eg. X_1 in Figure 1) while the distal position of the variable site was designated with the subscript '2' (eg. X_2 in Figure 1).

The genotypic frequencies for the wood and plains bison populations were calculated for each polymorphic site identified and were compared to Hardy-Weinberg proportions. The difference between the observed and expected values were tested using Chi-squares, corrected for small sample size (Levene, 1949).

The coefficient of inbreeding (f) within each population was calculated for each polymorphic restriction site following Barker et al. (1986).

The null hypothesis that f=0, was tested using Chi-squares for difference from zero (Barker et al., 1986).

Analysis of population structure follows the methods of Wright (1965). Wright's F-statistics are the correlations found between two uniting gametes within a population (F_{IS}) as well as in the species as a whole (F_{IT}). The correlation between two uniting gametes within a population, relative to gametes of the entire species, indicates the amount of genetic differentiation (F_{ST}) between the wood and plains bison. In this analysis, the F-statistics were calculated for polymorphic loci using the following formulae (Guries & Ledig, 1979):

[1] $$F_{IS} = 1 - H_i/[1 - 1/(2N_i + 1)]2p_iq_i$$

where H_i is the observed number of heterozygotes in the i^{th} subpopulation. The denominator is the expected number of heterozygotes corrected for finite population size.

[2] $$F_{ST} = \sigma^2_p/pq$$

where σ^2_p is the weighted sum of squared deviations of the individual subpopulation gene frequencies from the mean gene frequency, divided by the number of subpopulations (Guries & Ledig, 1979), and p and q are the weighted mean haplotype frequencies at the locus under consideration.

The correlation between two uniting gametes within the species

as a whole was derived from the F_{IS} and F_{ST} values:

[3] $$F_{IT} = F_{IS} + (1 - F_{IS})F_{ST}$$

The extent of homogeneity between the bison populations was estimated through the contingency Chi-square analysis (Feinberg, 1983).

Genetic variation between the two subspecies was determined through Nei's (1972) measures of genetic identity and genetic distance. The genetic identity (I, the proportion of identical genes between populations) was calculated using the formula:

[4] $$I = \sum P_{Xi}P_{Yi} / [(\sum P_{Xi})^2 (\sum P_{Yi})^2]^{1/2}$$

where P_{Xi} is the frequency of haplotype i in population X and P_{Yi} is the frequency of the same haplotype (i) in population Y.

The genetic distance (D, the estimated number of haplotype substitutions per locus) was computed using the formula:

[5] $$D = -\ln I$$

The genetic variation was estimated for all restriction sites identified.

2.3 RESULTS

The 22 enzyme/probe combinations employed in this study provided 28 fragments (50 restriction sites) for the examination of intra- and inter-population genetic variability (Table 2.1). Of these 28 fragments, two revealed restriction fragment length polymorphisms (RFLP's), both of which were associated with the growth hormone releasing factor (GHRF) locus. When digested with Eco RV, the bison GHRF locus yielded fragments that were either 8.9 kb or 14.4 kb; Sph I digestion of this locus yielded fragments that were either 11.1 kb or 12.6 kb in length (Figure 2.2). Each of these RFLP's were identified within both the wood and plains bison samples. That these fragments were polymorphic rather than multiple fragments from a single locus is revealed in Figure 2; individual bison could be either homozygous or heterozygous for each of the Eco RV and Sph I fragments.

A dimorphic restriction fragment was assumed to reflect the existence of a dimorphic restriction site: the two Eco RV restriction fragments arose from a dimorphic Eco RV restriction site (with positions designated as E_1 and E_2 according to the system of nomenclature presented in Figure 2.1); the two Sph I fragments arose from a dimorphic Sph I restriction site (with positions designated as S_1 and S_2 according to the system of nomenclature presented in Figure 2.1).

The frequencies of the Eco RV and the Sph I dimorphic sites are

presented in Table 2.2. Within wood bison, E_1 appeared at a higher frequency than E_2 while the reverse was true within the plains bison. At the dimorphic Sph I site, S_1 appeared at a higher frequency than S_2 within wood bison while S_1 and S_2 appeared at equal frequency within the plains bison.

When the genotypes are defined by combining the two restriction sites, Eco RV and Sph I, four haplotypes are possible: E_1S_1, E_2S_1, E_2S_2, and E_1S_2 (Table 2.3). Out of the four haplotype patterns one pattern, E_1S_2, may not be present in either the wood or plains bison samples.

The expected genotypic frequencies, calculated from the observed frequencies within the experimental animals, are presented in Table 2.2. The observed frequencies of the dimorphic sites did not differ from a Hardy-Weinberg equilibrium within either the wood or the plains bison. Although, a slight excess of heterozygotes resulted in the calculation of negative inbreeding coefficients for both the wood and plains bison (Table 2.4), the f value is not significantly different from zero ((Wood: Eco RV, χ^2=1.90, 1 d.f., $p \leq 0.05$; Sph I, χ^2=0.46, 1 d.f., $p \leq 0.05$)(Plains: Eco RV, χ^2=1.01, 1 d.f., $p \leq 0.05$; Sph I, χ^2, 1 d.f., $p \leq 0.05$)).

To analyze population structure within and between the two populations, the F-statistics were computed (Table 2.4).

The degree of homogeneity was significantly different between the wood and plains bison at both the Eco RV (χ^2=24.26, 1 d.f., p=0.0) and the Sph I (χ^2=11.17, 1 d.f., p=0.0) locus.

The genetic identity between the wood and plains bison was 0.985 which resulted in a genetic distance of 0.015. The genetic distance between the bison was greater at the Eco RV locus (0.622) than at the Sph I locus (0.199). A comparison of the North American bison GHRF-related restriction fragments with those identified in the four European bison samples is presented in Figure 2.3. Both Eco RV and Sph I identify GHRF-related restriction fragments that were also identified within the North American species. However, unlike the North American bison, the European bison is homozygous for each of the Eco RV and Sph I fragments. The European bison Eco RV restriction fragment is 14.4 kb while the Sph I restriction fragment is 11.1 kb.

2.4 DISCUSSION

2.4.1 Intra-population variability

As defined by restriction site loci associated with growth-regulating genes, the genetic diversity within both the wood and plains bison populations at Elk Island National Park (EINP) appears to be low; polymorphisms were identified at only 2 of the 50 restriction site loci examined. This low level of intra-population genetic diversity is compatible with the history of the two populations; in the establishment of both the plains and the wood population, a very limited number of animals were available. The animals presently in EINP result from an original stock of 22 wood bison and 30 plains bison (Ogilivie, 1979; Novakowski, 1989). Moreover, as a consequence of this limitation on the availability of foundation genetic material, the present representatives may not, in fact, reflect the genetic diversity which existed within the bison that once inhabited North America.

While very few restriction site polymorphisms were identified within the wood and plains bison, the distribution of the identified polymorphic sites does reveal several structural features of the existing EINP populations. The Hardy-Weinberg equilibrium of the restriction site genotypes implies that both bison populations are random mating; each male in the population appears to have an equal chance of mating with each female. The negative inbreeding coefficients further imply that the two EINP bison populations are

panmictic. Thus, although both populations have experienced bottlenecks in the past, our study suggests that the existing genetic information within each population is being evenly distributed.

The concept of random mating within each bison population may appear to be incompatible with the polygynous mating system typical of bison (Lott et al., 1987). The principal factor influencing this random distribution of genotypes is time. Both bison populations in EINP were established over 25 years ago, and this span encompasses the predominant lifetime of a bison. Within the polygynous mating system, selection for body size, weaponry, and behavior in males restricts monopolization of females to certain age classes (Jarman, 1983). Although the age at which male bison may breed is a selective factor, over a lifetime most bulls will eventually mate, thus facilitating random mating within the bison populations.

Population structure of the EINP bison may also contribute to the randomized arrangement of genotypes. With the exception of the breeding season, bison bulls remain either solitary or roam in small temporary groups; cows form larger groups with the calves and young bulls. As the mating season approaches, the cows congregate into larger breeding clusters where they are joined by the mature bulls (Lott, 1972; 1974a; 1979). Therefore, depending upon the size of the breeding cluster relative to the total population size, a small number of dominant bulls may have access to a large proportion of the breeding females; the inability of many less dominant bulls to contribute to the gene pool could thereby result in genotypic

frequencies which deviate from a Hardy-Weinberg equilibrium. However, Shackleton (1968) indicated that the group size of bison, within the wooded habitat of EINP, is smaller than it would be in open range. This smaller group size would increase the number of breeding clusters available. As a consequence, the less dominant bulls would have a greater probability to contribute to the gene pool, thereby increasing the opportunity to distribute genetic material uniformly throughout the population.

Random distribution of genotypes may also be attributed to the mobility of cows. Individual females are known to move rapidly and extensively through home ranges (Lott and Minta, 1983). This thorough population mixing would increase each cow's chances of encountering different bulls in various years. Moreover, since the bulls are not associated with a specific herd throughout much of the year, further distribution of available genetic material may result through the random distribution of bulls among the breeding clusters during each breeding season.

The Hardy-Weinberg distribution of the restriction site genotypes revealed within the two bison populations, also indicates that there is no strong selection for specific GHRF genotypes in either population.

2.4.2 Inter-population variability

While considerable controversy exists regarding both the taxonomic status of wood and plains bison, as well as their

evolutionary history (for review see McDonald, 1981), a consensus does exist that wood and plains bison evolved from a common ancestor (Wilson, 1969; Guthrie, 1970; Geist and Karsten, 1977; McDonald, 1981; van Zyll de Jong, 1986). The time of divergence of the two bison types has been estimated by Wilson (1969) to have occurred approximately 5000 years ago.

The low level of genetic diversity between the two populations indicates recent divergence. The genetic identity between the two populations (0.985) further emphasizes that the wood and plains bison are closely related. However, the F_{ST} value (0.23) together with the Contingency Chi-Square analysis, indicates that the frequencies of the identified polymorphic restriction sites differ significantly between the two populations. This significant difference is compatible with the suggestion that the wood and plains bison exist as two unique populations.

The degree of differentiation between the two bison populations is very similar to that which Ayala (1975) suggested would exist between two geographically isolated populations. Furthermore, the genetic identity between the two bison populations (0.985) corresponds closely to the identity (0.933-0.983 - based upon protein electrophoretic analysis) found in geographically isolated small mammal populations (Ayala, 1975).

The genetic differentiation between the bison populations (0.23) is much higher than the genetic differentiations (derived from protein electrophoretic analysis) reported for other geographically

isolated populations by Selander and Kaufman (1975); the highest differentiation cited by these researchers was 0.17 for interfarm house mouse populations. In terms of large mammals, Ryman et al. (1980) has reported a genetic differentiation of 0.096 for geographically isolated moose populations. Since nucleotide-based analysis would likely identify a greater degree of polymorphism than protein-based analysis, RFLP-derived genetic differentiations of these populations may, in fact, be very similar to that calculated for the bison populations.

Thus, our preliminary restriction site analysis suggests that the wood and plains bison populations at EINP have at least reached the stage of geographic isolation in their evolutionary divergence. The first step of the evolution (speciation) process is the isolation of populations by geographic barriers whereupon each population accumulates unique genetic differences. In the next step of geographic speciation, organisms are recognized as subspecies. At this level, differentiated populations have achieved at least partial reproductive isolation in which matings between individuals result in progeny with reduced fitness. The reproductive ability, exhibited by the hybrid bison in Wood Buffalo National Park, indicates the wood and plains bison have not yet obtained this stage in their evolutionary divergence. However, according to the guidelines of the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 1979), this classification of wood and plains bison as geographic populations would require that separate management of the two bison

types be continued.

Since the European wisent of the Old World had evolved before the North American bison, the homozygous fragment revealed within the Eco RV and Sph I wisent digests suggests this may be the ancestral bison allele. A considerable expansion of our molecular analysis of the wisent will be very valuable in further defining the taxonomic status of the wood and plains bison. While the actual time of divergence between this European bison and the North American bison remains uncertain (Wilson, 1969; Guthrie, 1970; Geist, 1977; McDonald, 1981), the wisent has been geographically separated for at least 10,000 years (McDonald, 1981). Fluctuations in sea level, caused by the freezing and thawing of glaciers, resulted in the appearance and disappearance of a land connection between eastern Siberia and Alaska. This land bridge made possible the migration of the European bison into North America; the Bering Land Bridge last joined Siberia and Alaska during the Late Wisconsin period (McDonald, 1981). On the basis of this geographic separation, and on the significant phenotypic differences between the European and North American bison (van Zyll de Jong, 1986), most researchers (for review see McDonald, 1981) appear confident in their classification of the wisent as a distinct species. An expanded examination of the nucleotide variation, which exists among these three bison types, would allow their degree of relationship with one another to be predicted; the degree of relatedness between wood and plains bison could then be considered relative to the relatedness between the North American and

European bison forms. This further molecular analysis of wood, plains and wisent populations, as well as of populations with clearly defined subspecific relationships, will undoubtedly be necessary to substantiate the subspecific designations of wood and plains bison.

2.5 REFERENCES

Avise, J.C., Lansman, R.A., Shade, R.O. 1979. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. I. Population structure and evolution in the genus Peromyscus. Genetics 92: 279-295.

Ayala, F.J. 1975. Genetic differentiation during the speciation process. Evol. Biol. 8:1-78.

Barker, J.S.F., East, P.D., Weir, B.S. 1986. Temporal and microgeographic variation in allozyme frequencies in a natural population of Drosophila buzzatii. Genetics 112:577-611.

Committee on the Status of Endangered Wildlife in Canada. 1979. Endangered Canadian Wildlife. Summary Sheets. Canadian Wildlife Service, Edmonton, Alberta, Canada.

Cronin, M.A. 1986. Genetic relationships between white-tailed deer, mule deer and other large mammals inferred from mitochondrial DNA analysis. M.Sc. Thesis. Montana State University, Bozeman, Montana.

Feinberg, A.P., Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analyt. Bioc. 132:6-13.

Feinberg, A.P., Vogelstein, B. 1984. Addendum to a technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analyt. Bioc. 137:266-267.

Feinberg, S.E. 1983. The analysis of cross-classified categorical

data. Second edition. The MIT Press. Cambridge, Massachusetts and London, England.

Ferris, S.D., Sage, R.D., Prager, E.M., Ritte, U., Wilson, A.C. 1983a. Mitochondrial DNA evolution in mice. Genetics 105:681-721.

Ferris, S.D., Sage, R.D., Huang, C.M., Nielson, J.T. Ritte, U., Wilson. A.C. 1983b. Flow of mitochondrial DNA across a species boundary. Proc. Natl. Acad. Sci. U.S.A. 80:2290-2294.

Froesch, E.R., Schmid, Chr., Schwander, J., Zapf, J. 1985. Actions of insulin-like growth factors. Ann. Rev. Physiol. 47:443-467.

Geist, V., Karsten, P. 1977. The wood bison (Bison bison athabascae Rhoads) in relation to hypotheses on the origin of the American bison (Bison bison Linnaeus). Z. Saugetierk, 42:119-127.

Gorbman, A., Dickhoff, W.W., Vigna, S.R., Clark, N.B., Ralph, C.L. 1983. The pars distalis, Multihormonal regulation of physiological adjustments In Comparative endocrinology. John Wiley & Sons, New York. pp. 125-136, 547-558.

Gordon, D.F., Quick, D.P., Erwin, C.R., Donelson, J.E., Maurer, R.A. 1983. Nucleotide sequence of the bovine growth hormone chromosomal gene. Mol. Cell. Endocrinology 33:81-95.

Guries, R.P., Ledig, T.F. 1979. Genetic structure of populations and differentiation in forest trees. Symposium on isozymes of North American forest trees and forest insects. Berkeley, California. Pp. 42-47.

Guthrie, R.D. 1970. Bison evolution and zoogeography in North America during the Pleistocene. The Quarterly Review of Biology 45:1-15.

Jarman, P. 1983. Mating system and sexual dimorphism in large, terrestrial, mammalian herbivores. Biol. Rev. 58:485-520.

Levene, H. 1949. On a matching problem arising in genetics. Ann. Math. Stat. 20:91-94.

Lott, D.F. 1972. Bison would rather breed than fight. Nat. Hist. 81:40-45.

Lott, D.F. 1974a. Sexual and aggressive behavior of mature male American bison. In Behavior in ungulates and its relation to management. Edited by V. Geist and F. Walthers IUCN, Morges, Switzerland pp. 382-394.

Lott, D.F. 1979. Dominance relations and breeding rate in mature male American bison. Z. Tierpsychol. 49:418-432.

Lott, D.F., Minta, S.C. 1983. Random individual association and social group instability in American bison (Bison bison). Z. Tierpsychol. 61:153-172.

Lott, D.F., Shaw, J.H., Stormont, C. 1987. Should public herds be trading bison to maintain diversity in the gene pool and/or prevent inbreeding depression? In Proceedings of the North American Bison Workshop, Sponsored by United States Fish and Wildlife and Glacier Natural History Association, Missoula, MT. Pp. 59-60.

Maniatis, T., Fritsch, E.F., Sambrook, J. 1982. Molecular cloning: a

laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor. N.Y.

Mayo, .E., Cerelli, G.M., Rosenfeld, M.G., Evans, R.M. 1985. Characterization of cDNA and genomic clones encoding the precursor: to rat hypothalamic growth hormone-releasing factor. Nature 314:464-467.

McDonald, J.N. 1981. North American bison, their classification and evolution. University of California Press, Berkeley, Los Angeles, London.

Nei, M. 1972. Genetic distance between populations. Amer. Nat. 106:283-292.

Novakowski, N.S. 1989. Historical origin of national park plains bison. Department of the Environment, Canadian Park Service, Natural Resources Branch, Ottawa, Ontario, Canada.

Ogilivie, S.C. 1979. The park buffalo. Calgary-Banff Chapter. National and Provincial Park Association, Canada.

Peden, D.G., Kraay, G.J. 1979. Comparison of blood characteristics in plains bison, wood bison, and their hybrids. Can. J. Zool. 57:1778-1784.

Powell, J.R. 1983. Interspecific cytoplasmic gene flow in the absence of nuclear gene flow: Evidence from Drosophila. Proc. Natl. Acad. Sci. U.S.A. 80:492-495.

Raisz, L.G., Kream, B.E. 1981. Hormonal control of skeletal growth. Ann. Rev. Physiol. 43:225-238.

Ramsey, P.R., Avise, J.C., Smith, M.H. Urbston, D.F. 1979.

Biochemical variation and genetic heterogeneity in South Carolina deer populations. J. Wildl. Manage. 43:136-142.

Reynolds, H.W., Glaholt, R.D., Hawley, A.W.L. 1982. Bison In Wild mammals of North America. Edited by J.A., Chapman, G.A., Feldhamer, G.A. pp. 972-1007.

Rotwein, P. 1986. Two insulin-like growth factor I messenger RNAs are expressed in human liver. Proc. Natl. Acad. Sci. U.S.A. 83:77-81.

Ryman, N., Reuterwall, C., Nygren, K., Nygren, T. 1980. Genetic variation and differentiation in Scandinavian moose (Alces alces): are large mammals monomorphic. Evolution 34:1037-1049.

Salmon, R.K., Berg, R.T., Yeh, F.C., Hodgetts, R. 1988. Identification of a variant growth hormone haplotype in mice selected for high body weight. Genet. Res. 52:7-15.

Selander, R.K., Kaufman, D.W. 1975. Genetic structure of populations of the brown snail (Helix Aspersa). I. Microgeographic separation. Evolution 29:385-401.

Shackleton, D.M. 1968. Comparative aspects of social organization of American bison. M. Sc. thesis, University of Western Ontario, London, Ontario.

Shen, L., Pictet, R.L., Rutter, W.J. 1982. Human somatostatin I: Sequence of the cDNA. Proc. Natl. Acad. Sci. U.S.A. 79:4575-4579.

Speiss, E.B. 1977. Genes in populations. John Wiley & Sons, New York.

Spolsky, C., Uzzell, T. 1986. Evolutionary history of the hybridogenetic hybrid frog Rana esculenta as deduced from mtDNA analysis. Molec. Biol. and Evol. 3:44-56.

van Zyll de Jong, C.G. 1986. A systematic study of recent bison, with particular consideration of the wood bison. National Museums of Canada. Publications in Natural Sciences. No. 6.

Wilson, W. 1969. Problems in the speciation of American fossil bison In Post-Pleistocene man and his environment on the Northern Plains. Proceeding of the first annual paleo-environmental workshop. Students' press, University of Calgary, Calgary, AB.

Wright, S. 1931. Evolution in Mendelian populations. Genetics 16:97-159.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19:395-420.

Wright, J.W., Spolsky, C., Brown, W.M. 1983. The origin of the parthenogenic lizard Cnemidophorus laredoensis inferred from mitochondrial DNA analysis. Herpetologica 39:410-416.

Ying, K.L., Peden, D.G. 1977. Chromosomal homology of wood bison and plains bison. Can. J. Zool. 55:1759-1762.

Zamora, L.E. 1983. An analysis of bison erythrocyte antigens and blood proteins. M. Sc. Thesis. Texas A & M University.

Zapf, J., Froesch, E.R. 1986. Insulin-like growth factors/somatomedins: structure, secretion, biological

actions and physiological role. Hormone Res. 24:121-130.

Table 2.1:

Restriction fragment lengths associated with growth-regulating loci.

Population	Enzyme	Locus[a]	Fragment Sizes (kb) Allele 1	Allele 2	Number of Restriction Sites
Wood/Plains:					
	Eco RI	GH	5.40		2
		IGF-I	9.80		4
			8.20		
			3.45		
		S	18.90		3
			3.20		
		GHRF	5.65		2
	Bam HI	GH	13.90		2
		IGF-I	15.10		3
			13.00		
		GHRF	14.00		2
	Pst I	GH	.64		2
		IGF-I	6.40		3
			5.90		
		GHRF	3.81		2
	Hind III	GH	20.50		2
		S	20.54		3
			8.00		
		GHRF	8.75		2
	Pvu II	GH	1.00		2
		S	21.00		2
	Xba I	GH	13.70		2
		S	8.70		2
		GHRF	3.85		2
	Bgl I	GHRF	7.00		2
	Dra I	GHRF	6.95		2
	Eco RV	GHRF	8.90 (E_1)	14.40 (E_2)	2
	Sph I	GHRF	11.10 (S_1)	12.60 (S_2)	2
European:					
	Eco RV	GHRF	14.40		
	Sph I	GHRF	11.10		

kb - kilobase

[a] Loci were designated by the following abbreviations: growth hormone, GH; insulin-like growth factor-I, IGF-I; somatostatin, S; growth hormone releasing factor, IGF-I.

Table 2.2:

Frequency of alleles at polymorphic loci in wood and plains bison.

Locus	POPULATION Wood	Plains
Eco RV		
N	20	20
E_1	0.75	0.20
E_2	0.25	0.80
Sph I		
N	20	20
S_1	0.85	0.50
S_2	0.15	0.50

Chi-square test for deviation from Hardy-Weinberg equilibrium.

Population	Locus	Genotype	Obs. Freq.	Exp. Freq.	Chi-Square	D.F.	P
Wood	Eco RV	E_1E_1	10	11.15			
		E_1E_2	10	7.69			
		E_2E_2	0	1.15	1.97	1	0.16
	Sph I	S_1S_1	14	14.39			
		S_1S_2	6	5.23			
		S_2S_2	0	0.39	0.51	1	0.48
Plains	Eco RV	E_1E_1	0	0.72			
		E_1E_2	8	6.56			
		E_2E_2	12	12.72	1.07	1	0.30
	Sph I	S_1S_1	4	4.87			
		S_1S_2	12	10.26			
		S_2S_2	4	4.87	0.61	1	0.44

Table 2.3:

Observed Genotypes at Sph I, Eco RV Polymorphic Sites

		SPH I		
		S_1S_1	S_1S_2	S_2S_2
ECO RV	E_1E_1	10 wood	0	0
	E_1E_2	4 wood 3 plains	6 wood 5 plains	0
	E_2E_2	1 plains	7 plains	4 plains

Table 2.4:

Inbreeding coefficient for wood and plains bison at each polymorphic site.

Population	Locus	Observed Heterozygotes	Expected Heterozygotes	Fixation Index (f)
Wood	Eco RV	10	7.69	-0.33
	Sph I	6	5.23	-0.18
Plains	Eco RV	8	6.56	-0.25
	Sph I	12	10.26	-0.20

Estimates of F_{IS}, F_{IT}, and F_{ST} for Two Polymorphic Sites in Bison bison.

Locus	F_{IS}	F_{IT}	F_{ST}
Eco RV	-0.295	0.098	0.303
Sph I	-0.192	-0.026	0.140
Ave. over Both sites	-0.241	0.040	0.227

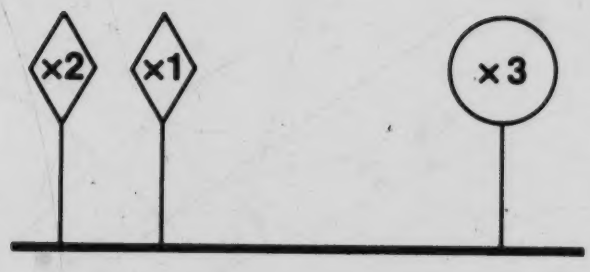

Figure 2.1. Relationship between identified dimorphic restriction fragments and the positions of the variable restriction site. A dimorphic restriction fragment was assumed to reflect the existence of a dimorphic restriction site. In each case where such a situation was assumed to exist, the position of the variable site most proximal to the monomorphic site (labelled X_3) was designated with the subscript '1' (eg. X_1) while the distal position of the variable site was designated with the subscript '2' (eg. X_2).

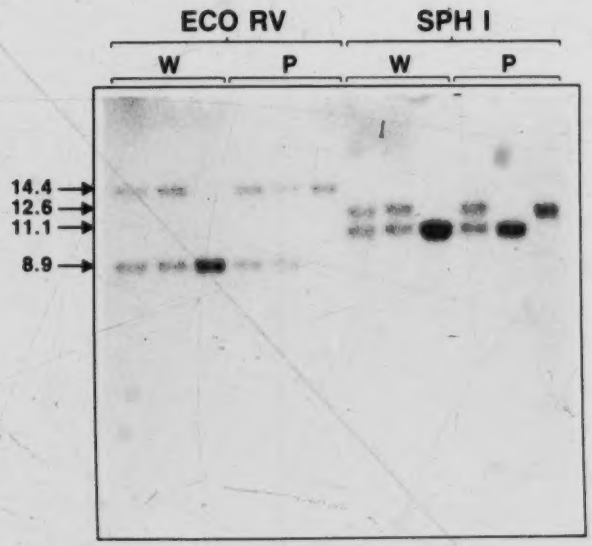

Figure 2.2. Autoradiogram of the GHRF-related RFLP's within the wood (W) and plains (P) bison. These polymorphic restriction fragments were revealed when bison DNA was digested with either Eco RV or Sph I. Individual wood and plains bison could be either homozygous or heterozygous for each of the Eco RV and Sph I restriction fragments.

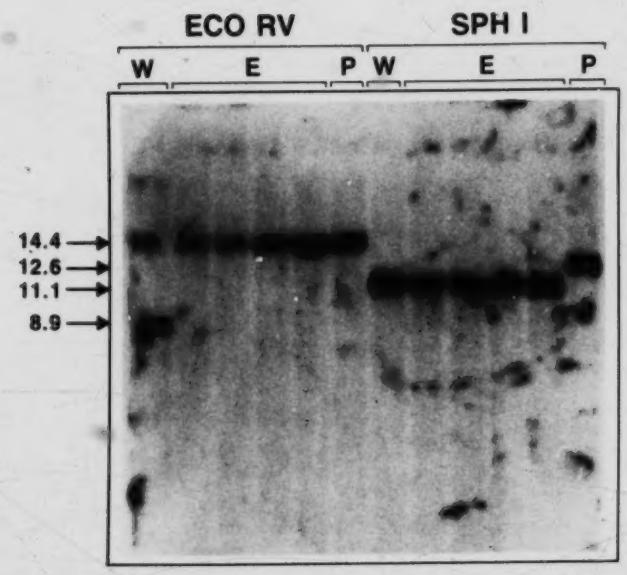

Figure 2.3. Autoradiogram of the European (E) and North American (wood (W) and plains (P)) bison GHRF-related restriction fragments. When European bison DNA was digested with Eco RV the GHRF locus yielded a restriction fragment of 14.4 kb and digestion with Sph I yielded a fragment of 11.1 kb.

III. IDENTIFICATION OF HIGH MOLECULAR WEIGHT DNA ISOLATED FROM A 36,000 YEAR OLD STEPPE BISON (B. PRISCUS)

3.1 INTRODUCTION

During the Pleistocene era, speciation and extinction rates quadrupled over that of the Tertiary (Geist, 1983), resulting in current confusion with respect to the evolution of mammals, particularly of bison. The fossil record, together with information on coexistent forms, indicates the evolutionary pattern of bison cannot be defined as either unidirectional, or as a series of invasions from the Old World to the New (Guthrie, 1970; McDonald, 1981). This unresolved puzzle has caused a heated controversy among many Pleistocene paleontologists (McDonald, 1981). The discovery of a 36,000 - year old steppe bison (Bison priscus) within the permafrost layers of Alaska (Guthrie, 1988) has created potential for applying molecular genetic techniques to resolve the bison dispute.

Recently, DNA from ancient remains has been successively isolated from a number of species including humans, sloths (Mylodon), and quagga (Equus quagga) (Paabo, 1985; 1989; Higuchi et al., 1984). Since the inter-relatedness of species is encoded within the genetic processes which are passed on from generation to generation, comparison of DNA sequences obtained from both this prehistoric bison and modern bison could be a direct and powerful method of resolving

this controversy.

In the extraction of ancient DNA, a common problem encountered is nucleic acid degradation which hinders the application of advanced molecular genetic techniques; moreover, another primary concern revealed by Johnson et al. (1985), in an attempt to isolate mammoth DNA, is the possible extraction of a foreign DNA resulting from microbial contamination. Therefore, prior to further molecular analysis, the nucleic acid extracted from prehistoric remains must be positively identified to prevent the comparison of unrelated DNAs.

The purpose of the study undertaken herein was therefore to isolate high molecular weight DNA from the prehistoric steppe bison tissue and to verify the identity of the extracted DNA.

3.2 MATERIAL AND METHODS

3.2.1 DNA extraction and purification

Dry muscle from a prehistoric bison (Bison priscus) was generously supplied by Dr. R.D. Guthrie from the University of Alaska. Owing to the inadequacy of conventional DNA extraction techniques, nucleic acid extraction from the ancient tissue followed a modified procedure of Johnson et al. (1985). Prior to rehydration in TEN buffer (10mM Tris-Cl (pH 7.5)/1mM EDTA (pH 8.0)/10mM NaCl) (Johnson et al., 1985), individual muscle fasciculi were separated and cut into smaller fragments. Ten ml of TEN buffer were used for each gram of tissue undergoing DNA isolation. Following a 12 to 16 h incubation at 37°C, the tissue/buffer mixture was homogenized by freezing in liquid nitrogen and subsequent grinding with mortar and pestle.

To facilitate cell lysis, proteinase K and sodium dodecyl sulfate (SDS) were added to the ground tissue at a final concentration of 40 ug/ml and 0.5%, respectively. This solution was then gently mixed on a multipurpose, vertical rotor for 16 to 20 h at 20°C. To separate the DNA from the tissue, the solution was centrifuged on a JA-20 (Beckman) rotor at 10,000 rpm for 1 h. The extracted supernatant was stored between 0°C and 4°C until purification, and the precipitate was again subjected to this sequence of grinding, lysing, mixing and centrifugation. This sequence of manipulation was repeated until the tissue had completely dissolved in the lysis

solution (approximately 6 or 7 cycles). The supernatants collected between manipulations were placed in separate tubes and were subjected individually to DNA purification.

For purification of DNA, the supernatant was extracted twice with an equal volume of TE (10mM Tris-Cl (pH 8.0)/1mM EDTA (pH 8.0)) - saturated phenol, followed by an extraction with 1:1 phenol/chloroform and finally with 24:1 chloroform/isoamyl alcohol. Isolation of DNA from the purifying agents was accomplished by centrifugation at 10,000 rpm for 5 min. The final aqueous phase was adjusted to 0.25 M sodium acetate (pH 5.2) and precipitated overnight with 2.5 volumes of 95% ethanol at -20°C. The resulting pellet was washed with 70% ethanol and resuspended in 100 ul of TE. The nucleic acid solution was then treated with 80 ug/ml ribonuclease A at 37°C for 3 h. Removal of ribonuclease A was acheived by extracting with equal volumes of phenol, 1:1 phenol/chloroform, and 24:1 chloroform/isoamyl alcohol, respectively; the DNA was subsequently precipitated in 0.25 M sodium acetate and 2.5 volumes of 95% ethanol. Following a 70% ethanol wash, the total DNA was further purified by elution through an Elutip-d column (Schleicher & Schuell, Montreal QB).

To evaluate the condition of the isolated DNA, the sample was electrophoresed on a 0.7% agarose gel in a 0.04 M Tris-acetate/0.001 M EDTA (TAE) (Maniatis et al., 1982) buffer at 30 v for 3 h (Figure 3.1). The gel included uncut human DNA, as well as DNA markers of known fragment size: Bacteriophage lambda cleaved with Hind III;

pBR322 cleaved with Sau3A I; high molecular weight (HMW) (BRL) marker. The size of the marker fragments ranged from 48.0 kb to 0.36 kb to allow for an estimation of prehistoric DNA size. For visualization of DNA, ethidium bromide (0.3 ug/ml) was added to the gel. The prehistoric DNA was also subjected to restriction endonuclease digestion and electrophoresed on a 0.7% agarose gel to further determine the quality of the DNA. Test digests of the DNA were performed and examined before and after chromatography to evaluate the effect of the Elutip-d column (Figure 3.2). Quantitation of the ancient DNA was determined by spectrofluorimetry.

3.2.2 Satellite band isolation

To determine the origin of the prehistoric DNA, a bison satellite band was isolated and employed as a probe. To obtain the satellite band, approximately 10 ug of plains bison DNA was digested with the restriction endonuclease Pst I (Bethesda Research Laboratories, Burlinton ON). The digested DNA was electrophoresed on a 1% LMP agarose (BRL) gel in TAE buffer at 30 v for 9 h with markers ranging from 48.0 to 0.36 kb. The 700 bp satellite band identified by its intensity (Figure 3.3), was manually cut from the gel with a scalpel, and the DNA was further purified of LMP agarose by way of the GeneClean procedure (BIO/CAN Scientific Inc., Mississauga ON). The concentration of the satellite DNA was determined by spectrofluorimetry.

3.2.3 Dot Blot and Labelling

To confirm the identity of the ancient DNA, a dot blot of the prehistoric bison was completed with the following DNA controls: plains bison, cow, human, drosophila, and Escherichia coli. The affinity of the bison satellite probe to the various DNAs was estimated by varying the amount of DNA loaded. The amounts (ug) of DNA added were: 0.02, 0.03, 0.05, 0.1, 0.3, 1.0 (Figure 3.4). The dot blot procedure followed the instructions outlined by GeneScreen Plus (New England Nuclear Research Products, Boston MA).

To acquire a sufficient amount of probe, 150 ng of satellite DNA were radiolabelled with [^{32}P] dCTP (ICN Biomedicals Canada Ltd., Montreal QB) by the random primer method (Feinberg & Vogelstein, 1983; 1984), and the labelled probe was separated from the unincorporated radionucleotides by spun column chromatography (Maniatis et al., 1982). Prehybridization, hybridization, and washing of the membrane followed the formamide procedure recommended by the supplier (NEN). The membrane was autoradiographed at -70^{o}C, for one hour, using Kodak GBX-2 film (Eastman Kodak Co., Rochester NY) and Lightning Plus intensifying screens.

3.3 RESULTS

Nucleic acid extraction and purification from the prehistoric bison was performed on two separate occasions. Although the technique employed and the amount of tissue used was identical, the total amount of DNA obtained from each tissue sample varied considerably, with 21 ug and 2 ug of DNA extracted, respectively. In the second extraction a greater amount of connective tissue was present and consequently resulted in a lower concentration of DNA.

As had been observed by Johnson et al. (1985), a brown colored impurity was present in the DNA collected and was still prominent after chromatography. The intensity of the impurity lessened with each successive manipulation of grinding, lysing, and centrifugation; as well, the concentration of DNA within samples of repeated manipulations increased up to the fifth extraction and decreased thereafter.

The condition of the bison DNA determined from gel electrophoresis revealed high molecular weight DNA (>45 kb) with very little visible shearing (Figure 3.1). Also, the RNA obtained was in excellent condition, with the two major rRNA bands distinctly visible within the gel. Those samples with the highest concentration of the colored impurity revealed a light blue hue within the gel. Column purification caused partial shearing of the prehistoric DNA, and it was found that the amount of shearing was directly related to the rate at which the DNA was forced through the column.

When the prehistoric DNA was cut with Eco RI before chromatography, zero to partial digestion resulted; digestibility was greater within samples taken from later extractions. Purification by the Elutip-d column increased digestibility in all samples; following passage over the column, Eco RI cleaved the DNA into multiple fragments ranging from approximately 25 kb to 1 kb (Figure 3.2). However, the digest pattern of the prehistoric DNA varied considerably from the Eco RI pattern of a modern bison.

The affinity of the bison satellite probe to the various DNA blots decreased as follows: bison, cow, prehistoric bison, E. coli, human, and drosophila (Figure 3.4). Hybridization of the satellite band to the human and drosophila blots was negligible. The signal detected within the 0.3 ug prehistoric dot was equivalent to the signal given by 0.02 ug of modern bison, a ratio of 1:15 between modern and prehistoric bison. Furthermore, when E. coli DNA is probed with bison satellite DNA, at least 20 times more microbial DNA is required to give the same signal as B. priscus DNA.

3.4 DISCUSSION

The DNA isolated from the prehistoric bison was greatly modified both, intrinsically and extrinsically, from that DNA isolated from extant species. Physically, the most discernible characteristic of the prehistoric DNA is a brown colored impurity which may represent Maillard products of reducing sugars (Paabo, 1989). Although the Maillard reaction generally involves heat, it has been shown that some freeze-dried foods, which have not been subjected to heat, are susceptible to this type of browning (Reynolds, 1965). The blue hue within the gel, which was particularly apparent within earlier extractions, was probably a result of this brown pigment. Johnson et al. (1985) had also discovered detectable amounts of a material that absorbs at visible wavelengths, and considered it to be a chemical modification of the ancient tissue.

Another alteration typical of ancient DNA is a size reduction to an average of a few hundred base pairs (Higuchi et al., 1984; Paabo, 1985; Paabo et al., 1988; Paabo, 1989; Paabo et al., 1989); the molecular size of the DNA extracted from B. priscus is much higher than the average DNA size extracted from other ancient remains. The extent of DNA size reduction is not correlated to the age of the samples but to the rapidity with which the body has been dessicated immediately after death (Paabo, 1989). The oxidative damage that occurs postmortem appears to reach a plateau in a relatively short time, after which it has only minimal additional effects (Paabo,

1989). The excellent condition of the prehistoric bison DNA indicates the carcass must have undergone rapid freezing, which hindered the processes of oxidation.

Chromatography of the bison DNA removed a portion of the colored product and also improved restriction enzyme digestibility. The correlation between the amount of impurity prevalent within the sample, and the cutability of the enzyme suggests the pigment has an influence on enzyme performance. Paabo et al. (1988) had also commented on an unidentified component within DNA extracts of 7,000 year old humans, which inhibited the activity of polymerase within the polymerase chain reaction. This inhibition was overcome by the addition of bovine serum albumin and increased amounts of enzyme.

An intrinsic modification of the prehistoric DNA was revealed upon restriction enzyme digestion, after which the prehistoric digest pattern varied considerably from that of modern bison. This variation is probably a consequence of DNA damage, a common occurrence within ancient nucleic acids. The nucleic acid extracted from B. priscus appeared as intact, high molecular weight DNA with very little visible shearing. However, depurination, a frequent process of DNA deterioration, may have resulted in nicks (missing bases), which cannot be detected by standard electrophoretic techniques. Such nicks are 'weak' points within the DNA, and through enzyme digestion they will break resulting in an indiscriminate range of fragments. The rate of depurination within native DNA at 37°C and pH 7.4 is 3×10^{-11} per second per cell (Lindahl and Nyberg,

1972); consequently, at this high activity, depurination would have occurred within the bison carcass regardless of freezing time. To determine whether nicks are present before digestion, the ancient DNA could have been subjected to pulse electrophoresis. Under this type of manipulation, weak points resulting from AP (apurination/apyrimidation) sites would separate revealing the condition of the DNA. In addition, DNA lesions such as baseless sites, oxidized pyrimidines, and cross links are alkali sensitive; therefore, treatment of the prehistoric bison DNA with alkali, along with treatment of a control DNA, would also give an indication of its condition (Paabo et al., 1988).

In past studies, DNA sequences longer than 500 base pairs have invariably proved to originate from contamination by modern DNA (Paabo et al., 1989); however, hybridization of the bison satellite probe to the prehistoric DNA reveals that a bison-related DNA exists within the prehistoric sample. The prehistoric and plains bison signal ratio of 1:15, indicates there is at least 1 ug of bison-related DNA per gram of prehistoric bison tissue. Chemical modifications within the prehistoric DNA may have influenced the binding efficiency of the probe. Further research is required to assess the affect of intrinsic/extrinsic DNA alterations on the accuracy of such molecular application. The inability of the satellite band to bind to the human blots indicates the probe is quite specific for bovine, which corroborates the validity of this experiment. Although, partial hybridization between bison and the E.

coli blots had occurred, this may have been a result of partial DNA sequence homology. A former dot blot that followed identical procedures to this study, revealed no hybridization between the bison satellite band and 5 ug of E. coli DNA. Therefore, binding may also be a result of technical variation or some source of outside contamination.

The identification of a bison-related DNA within B. priscus allows for further comparison of the genetic constitution of this prehistoric bison and modern day bison. The excellent condition of the nucleic acid extracted from the ancient bison increases the chances of successfully applying advanced molecular biological techniques. Analysis of DNA sequences of both extinct and extant species may be used to resolve the identity and relationship of bison and perhaps conclude the evolutionary pattern of this species.

3.5 REFERENCES

Feinberg, A.P., Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Bio. 132:6-13.

Feinber, A.P., Vogelstein, B. 1984. Addendum to a technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Bio. 137:266-267.

Geist, V. 1983. On the evolution of Ice Age mammals and its significance to an understanding of speciations. Walton Distinguished Lecture Series, Mountain Lake Biological Station 30:109-133.

Guthrie, R.D. 1970. Bison evolution and zoogeography in North America during the Pleistocene. The Quarterly Review of Biology 45:1-15.

Guthrie, M.L. 1988. Blue Babe: A story about a steppe bison mummy from Ice Age Alaska. White Mammoth, Fairbanks, Alaska.

Higuchi, R., Bowman, B., Freiberger,M.,Ryder, O.A., Wilson, A.C. 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature 312:282-284.

Johnson, P.H., Olson, C.B., Goodman, M. 1985. Prospects for the molecular biological reconstruction of the woolly mammoth's evolutionary history: isolation and characterization of deoxyribonucleic acid from the tissue of Mammuthus primigenius. Acta. Zool. Fennica 170:225-231.

Lindahl, T., Nyber, B. 1972. Rate of depurination of native deoxyribonucleic acid. Biochemistry 11:3610-3618.

Maniatis, T., Fritsch, E.F., Sambrook, J. 1982. Molecular cloning:a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y.

McDonald, J.N. 1981. North American bison, their classification and evolution. University of California Press, Berkeley, Los Angeles, London.

Paabo, S. 1985. Molecular cloning of ancient Egyptian mummy DNA. Nature 314:644-645.

Paabo, S., Gifford, G.A., Wilson, A.C. 1988. Mitochondrial DNA sequences from a 7000 year old brain. Nucleic Acids Research 16:9775-9787.

Paabo, S. 1989. Ancient DNA: extraction, characterization, molecular cloning and enzymatic amplification. Proc. Natl. Acad. Sci. U.S.A. 86:1939-1943.

Paabo, S., Higuchi, R.G., Wilson, A.C. 1989. Ancient DNA and the polymerase chain reaction. J.Biol. Chem. 264:9709-9712.

Reynolds, T.M. 1965. Chemistry of nonenzymic browning. Adv. Food Res. 14:167-283.

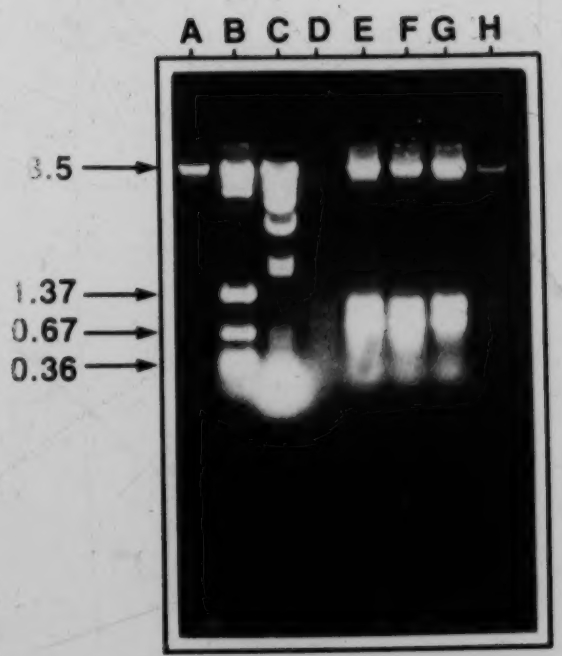

Figure 3.1. DNAs of A: human; B: high molecular weight marker; C: Lambda hind marker; D to H: prehistoric bison prior to RNasing. Designations D to H are DNAs isolated from the prehistoric bison from the second to sixth extraction, respectively. The yield of DNA within these samples is greatest in the intermediate extractions (E,F,G) and lowest in the earlier and later (D and H) extractions. The prehistoric DNA is similar in size to the human DNA (>45 kb), and the RNA obtained is in excellent condition with the two major rRNA bands distinctly visible.

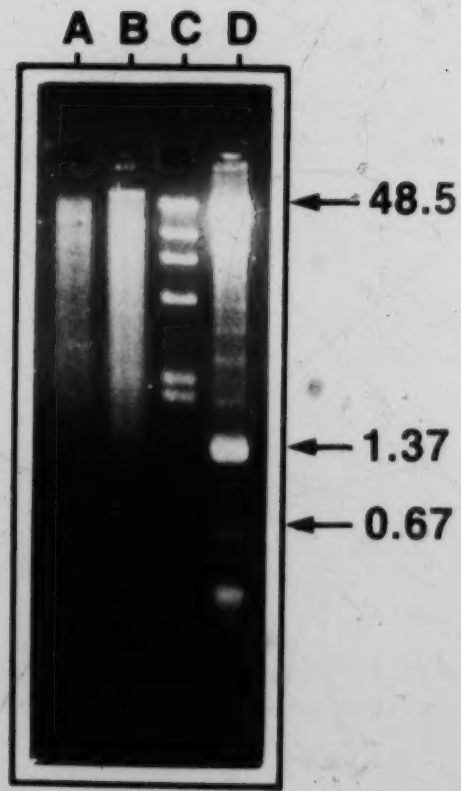

Figure 3.2. Column purified prehistoric bison DNA and plains bison DNA were digested with Eco RI and electrophoresed on a 0.7% agarose gel. A: prehistoric; B: plains bison; C: Lambda hind marker; D: high molecular weight marker. Purification by chromatography had increased digestibility of the prehistoric bison DNA, resulting in fragments ranging from 25 kb to 1 kb; however, the digest pattern differs from that of the plains bison.

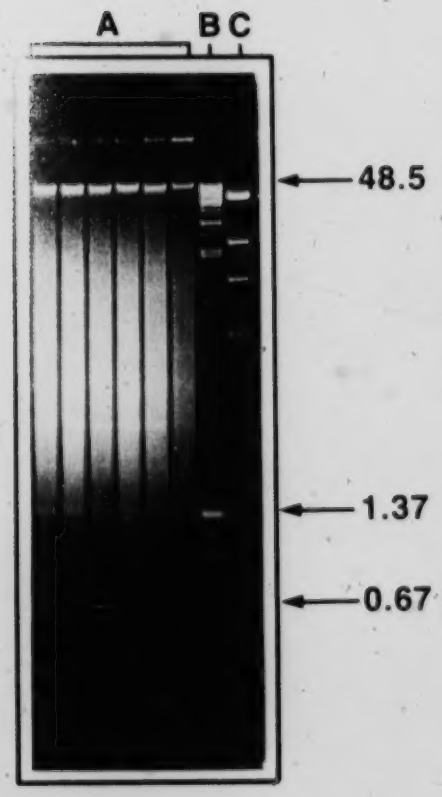

Figure 3.3. DNA of plains bison was digested with Pst I and electrophoresed on a 1% low melt point (LMP) gel, to facilitate the isolation of the 700 bp satellite band. A: Pst I restricted plains bison; B: high molecular weight marker; C: Lambda hind marker.

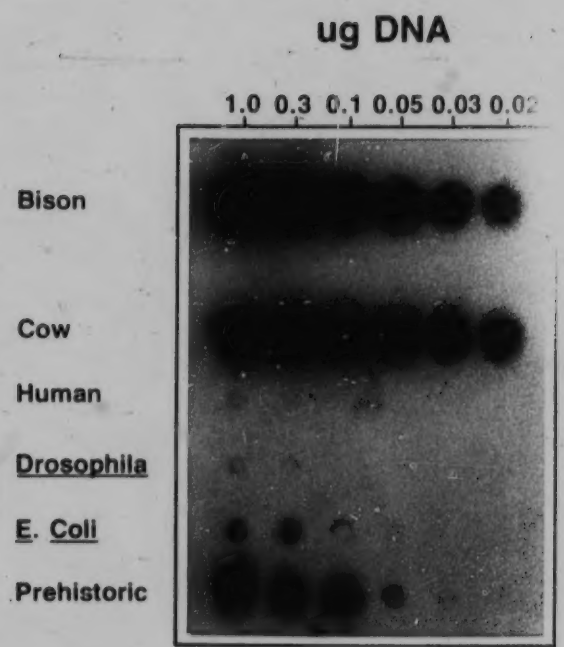

Figure 3.4. Dot blot which reveals the affinity of the bison satellite band to the prehistoric bison DNA and to other control DNAs. This dot blot shows that the prehistoric bison is related to bison and cow but not to human, Drosophila or E. coli.

GENERAL DISCUSSION

Theoretical considerations

The application of molecular biological techniques, to population and taxonomic questions, remains in its infancy. As a result, determination of the taxonomic status of a specific population, through the use of some form of molecular analysis, must await similar molecular examination of other populations with varying degrees of relatedness. Only through the assembly of a databank, which deals with the relationship between molecular genetic diversity and taxonomic classes, will molecular comparisons between populations become valuable in answering taxonomic questions.

Furthermore, comparisons between molecular studies will remain extremely difficult unless a common molecular technology is employed. Until recently, many studies (eg. Canham and Cameron, 1972; Bunch and Valdez, 1976; Avise et al., 1979; Ramsey et al., 1979; Soldal and Staaland, 1979; Gyllenstein et al., 1980; Chesser et al., 1982; Chesser, 1983; Fleishmann, 1986) have utilized the molecular analysis of proteins as a measure of genetic variability/diversity. Since variation at the protein level may differ from variation at the nucleotide level, comparisons between protein-based and nucleotide-based studies will allow only very general conclusions to be drawn. In an analysis of the electrophoretic variability of serum esterases in the lesser snow goose, Bargiello (1977) found the average heterozygosity of these

loci to be only 1.25%. However, an RFLP analysis conducted in the same species detected polymorphisms in every sequence probed (Quinn and White, 1987).

Moreover, the degree of nucleotide variability identified in a population may vary depending upon the specific sequence analyzed. Due to greater evolutionary constraints, protein-coding sequences as well as their associated regulatory elements will likely exhibit much lower levels of variability than non-coding sequences such as those examined by Quinn and White (1987). Moreover, variable rates of mutation within the genome (Britten, 1986) will also influence the degree of nucleotide variability identified within specific sequences.

Many of the restriction sites identified within the wood and plains bison study may lie within sequences involved in gene regulation. That most probes revealed only a single fragment per enzyme digest suggests that many of the identified restriction sites occurred within sequences which flank the protein-coding regions of the specific genes examined; the 5' flanking sequences of eucaryotic genes have been shown to contain important regulatory elements which often extend 10,000 base pairs upstream from the start site of transcription (Hatzopoulos et al., 1988). The possible occurrence of restriction sites within regulatory elements may be responsible for the low level of restriction site variability identified within the EINP park bison populations, relative to the variability found by Quinn and White (1987) within the snow goose.

The length of the probes (9.0 to 15.5 kb) employed by Quinn and White (1987), in comparison to the length of probes used in our study (0.34 to 1.14 kb), may have also contributed to the greater degree of nucleotide variability identified by these researchers. Theoretically, the number of restriction sites revealed per digest should increase with increasing probe length.

While both the specific type of genomic sequence examined (i.e. protein-coding versus non-coding), and the length of probes employed, differed between this study and that of Quinn and White (1987), both studies did utilize probes which were complementary to unique DNA sequences. However, probes which are complementary to repetitive genomic sequences may also be effective in the differentiation of species and in the determination of evolutionary relationships. Repetitive genetic elements, such as satellite DNA sequences, consist of many tandem copies of a single DNA sequence which are dispersed throughout the genome (Taparowski and Gerber, 1982). As in unexpressed unique DNA sequences, the copies of satellite DNA are less subject to selective pressures which limit the accumulation of restriction site polymorphisms (Taparowski and Gerber, 1982); moreover, since the satellite sequences are dispersed throughout the genome, the sequences are capable of evolving restriction sites independently of one another. As a consequence of this independent evolution of restriction sites, satellite probes may reveal multiple bands per enzyme digest, thereby increasing the probability of identifying polymorphic restriction sites. A study of satellite DNA

banding patterns among six species of whales (Arnason et al., 1988), which identified a species-specific hybridization pattern, emphasizes the significance of satellite probes in species determinations. Furthermore, an RFLP study of repetitive DNA in species of deer has revealed banding patterns which may be associated with the evolutionary relationships among the species (Lima-de-Faria et al., 1984).

However, while the multiple bands identified by satellite probes (and by large non-coding probes) may be very effective in uncovering genetic variation, such probes would be less effective for population analysis since multiple bands per digest would prevent the establishment of allelism (Hill, 1987). As a result, parameters associated with population structure, inbreeding and genetic distance (which are based upon allele frequencies) could not be determined.

Another genetic approach which would also be effective in revealing genetic variation and has been used in many studies (Avise et al., 1979; Cronin et al., 1988; Becker et al., 1988) to make taxonomic inferences is the analysis of mitochondrial DNA (mtDNA). As a result of the mitochondrion's inefficient repair mechanism (relative to the nucleus) the nucleotide sequences of mtDNA evolve approximately ten times faster than those in single copy nuclear genes (Becker et al., 1988). Within mtDNA there is a major noncoding region known as the D-loop (displacement loop), which is concerned with the initiation of DNA replication. The D-loop is particularly useful for the differentiation of closely-related species since the

extent of divergence within this region is at least ten times faster than for the mtDNA as a whole (Wilson et al., 1985). Although, the haploid nature of mtDNA prevents the establishment of allelism, an estimate of the genetic divergence between populations and species can be determined. As well, the maternal mode of inheritance of mtDNA makes it useful for studying maternal relationships and introgressive hybridization.

The utility of these various genetic parameters for taxonomic differentiation will only be established through further investigation. Not until a more complete understanding of these genetic parameters is obtained will a consensus be reached as to which molecular application is best suited for species and population differentiation. Further research will then be required to establish a molecular database for identification of various wildlife populations and species.

Evolutionary implications

The controversial history of the bison has not only created skepticism toward the bison's taxonomic status, but has also created a 'stir' among evolutionary scientists. As a result of the ambiguous prehistoric history of the bison, several theories have been proposed to account for a variety of bison phylogenies. The evolutionary relationship of the present-day bison, namely, B. bonasus, B. b. bison, and B. b. athabascae, and the inquiry into which of these three bison forms is the most evolved remains unresolved. Over the past 30 years molecular geneticists have devoted much effort into the

study of molecular evolutionary change and since then the association between species change and molecular change has revolutionized renown phylogenies.

An important discovery made in the study of molecular evolution is the approximate steady rate of nucleotide substitution within the DNA molecule (molecular evolutionary clock) (Nei, 1987; Wilson et al., 1987). Since molecular data reveal a more regular pattern of evolutionary change than morphological characters, they provide a clearer picture of the evolutionary relationships of organisms (Nei, 1987). However, within molecular genetics the evolution of a species is the result of one specie diverging into two new species (Nei, 1987). As well, every corresponding gene has an equal probability of mutating (evolving), therefore, two species diverging from a single lineage will be equally distant from the common ancestor. Therefore, rather than attempting to determine which form is the most evolved, one can specify in terms of molecular genetics 'which one of the three bison forms diverged prior to the other two'.

To obtain an estimate of the divergence time for the extant bison, DNA sequencing and comparison of the nucleotide substitutions between the bison would provide an indication of their genetic relationship. Comparison of a specific gene within Bison to the corresponding gene within a reference specie which lies phylogenetically outside of Bison (eg. Bos) would give an indication of the bison's true relationship. Inclusion of an outgroup organism such as Bos provides a root, thereby revealing which bison form

diverged the earliest; the bison type whose DNA sequence has the least number of nucleotide changes to Bos will have diverged prior to the other two bison forms.

Reflecting back to prehistoric times, the placement of the steppe bison, B. priscus, within the bison evolutionary tree is uncertain and of great controversy. Generally it is believed that B. priscus is ancestral to the present-day bison (McDonald, 1981; Wilson, 1969); however, based on the dispersal theory, Geist (1971) believes B. bonasus is ancestral to both B. priscus and B. bison. Through similar molecular genetic techniques involving DNA sequencing, such theories, as the dispersal theory, could either be supported or discounted. Sequence comparisons of the three extant bison, the prehistoric steppe bison as well as Bos (outgroup) can determine whether or not B. priscus diverged prior to the split of the three extant forms. If B. priscus had diverged after the split of the extant forms, genetic analysis can provide an estimate of its divergence time. On the other hand, if B. priscus diverged earlier, DNA sequencing can specify whether the prehistoric bison is a direct ancestor (same lineage) or an indirect ancestor (another lineage) of the three present-day bison.

The ideal region to examine evolutionary change is within the mitochondrial genome since it carries within its sequence the history of its lineage not complicated by recombination (Wilson et al., 1985). Mitochondrial DNA is also of great interest to evolutionary geneticists because of its high rate of evolution and maternal mode

of inheritance, which makes it ideal for studying ancestor-descendant relationships (Wilson et al., 1985; Paabo et al., 1989). Within molecular evolution, it is ideal to study those processes that occur rapidly enough to be studied on a time scale of 10^4 (Paabo et al., 1989); the slow accumulation of nucleotide substitutions found in the nuclear genome does not allow for phylogenetic construction of closely related species. Mitochondrial DNA is present in many copies per nucleated cell and this facilitates its survival and retrieval, an important aspect especially since degradation of ancient DNA is a common hindrance within molecular evolutionary genetics. Mitochondrial DNA is also uniformly distributed throughout the animal kingdom and is remarkably uniform in gene content (Wilson et al., 1985) which makes it ideal for species comparisons.

Within mtDNA there are three sequences that can be used for evolutionary study: cytochrome b, 12s rRNA, or the D-loop. The sequence chosen for examination depends largely on the degree of similarity between the species undergoing analysis. When the species being compared are more distant from one another a more slowly evolving sequence is used for analysis. In the construction of the DNA phylogeny of the extinct marsupial wolf (Thylacinus cynocephalus), comparisons were made to other distantly related marsupials such as the South American opposum (Philander) and the Australian tiger cat (Dasyurus). In this particular study the 12s rRNA gene was used for comparison since it evolves approximately five times slower than the cytochrome b gene (Thomas et al., 1989). A

slower evolving sequence is used to study distantly related species to reduce the chances of multiple substitutions; whereas in more closely related species, the cytochrome b gene is commonly used for the construction of phylogenetic trees. A more rapidly evolving sequence, such as cytochrome b, would be necessary for the comparison of Bison since they are classified within the same genus. However, since B. b. bison, B. b. athabascae and perhaps B. bonasus appear to be quite homogeneous, sequencing and comparison of the D-loop may be a better approach since its extent of divergence is ten times faster than the mitochondrial DNA as a whole. The D-loop appears to be an appropriate region for estimating divergence; however, it is a relatively new discovery and is still undergoing investigation to determine its potential for phylogenetic construction.

Once a sequence has been selected for phylogenetic construction, a new and powerful procedure for 'in vitro cloning' can be used to enhance the efficiency of determining the relationships between extinct and extant species. This novel technique is known as the polymerase chain reaction (PCR) and it is capable of selectively amplifying unique DNA segments within a few hours (White et al., 1989). The enzymatic amplification of a specific DNA segment involves the binding of two known synthetic oligonucleotide primers to sequences flanking the target segment (Kocher et al., 1989; White et al., 1989). These primers facilitate the binding of a DNA polymerase which then copies the segment. The PCR can synthesize many copies of a rare DNA sequence in the presence of a large number

of damaged molecules (Thomas et al., 1989), which makes this technique useful for the amplification of ancient DNA. Consequently, one can trace the changes in gene frequency through time by examining and comparing the nucletide changes between museum specimens and modern representatives of a population (Kocher et al., 1989). This rapid and sensitive procedure can also be used for taxonomic purposes to amplify target sequences which differentiate species and populations. Since the polymerase chain reaction can selectively amplify DNA sequences it can extend the resolving power and phylogenetic range of comparative studies; a sequence amplified with highly evolutionary conserved primers (universal primers) would provide a consistent metric for the comparison of populations and phyla (White et al., 1989).

At present, the classification and the evolutionary relationship of a species is based on morphological and phenotypic characters; however with the recent advances made in molecular technology, the use of a genetic parameter to assist in determining the identity and associations of wildlife species is close at hand. Whether or not molecular analysis will become the primary or sole factor for species differentiation, is a developing controversy within the system of taxonomic classification. By itself or in association with other parameters, genetic analysis has become an important tool within wildlife management, and through the continued efforts of molecular geneticists the association between the genetic constitution and the phenotype of an organism will eventually be more clearly understood.

REFERENCES

Arnason, U., Allderdice, P.W., Lien, J., Widegren, B. 1988. Highly repetitive DNA in the baleen whale genera Balaenoptera and Megaptera. J. Mol. Evol. 27: 217-221.

Avise, J.C., Smith, M.H., Selander, R.K. 1979. Biochemical polymorphism and systematics in the genus Peromyscus. VII. Geographic differentiation in members of the truei and maniculatus species groups. J. Mamm. 60:177-192.

Bargiello, T.A., Grossfield, J., Steele, R.W., Cooke, F. 1977. Isoenzyme status and genetic variability of serum esterases in the lesser snowgoose, Anser caerulescens caerulescens. Bioch. Gen. 15:741-763.

Becker, I.I., Grant,W.S., Kirby, R., Robb, F.T. 1988. Evolutionary divergence between sympatric species of Southern African Hakes, Merluccius capensis and M. paradoxus. II. Restriction enzyme analysis of mitochondrial DNA. Heredity 61:21-30.

Britten, R.J. 1986. Rates of DNA sequence evolution differ between taxonomic groups. Science 231:1393-1398.

Bunch, T.D., Valdez, R. 1976. Comparative morphology, chromosome types, transferrins and hemoglobins of Iranian and North American desert wild sheep. Desert Bighorn Council 1976 Transactions 20:13-24.

Canham, R.P. Cameron, D.G. 1972. Variation in the serum proteins of the red-backed mice Clethrionomys rutilus and C. gapperi and

its taxonomic significance. Can. J. Zool. 50:217-227.

Chesser, R.K., Reuterwall, C., Ryman, N. 1982. Genetic differentiation of Scandinavian moose Alces alces populations over short geographical distances. Oikos 39:125-130.

Chesser, R.K. 1983. Genetic variability within and among populations of the black-tailed prairie dog. Evolution 37:320-331.

Cronin, M.A., Vyse, E.R., Cameron, D.G. 1988. Genetic relationships between mule deer and white-tailed deer in Montana. J. Wildl. Manage. 52:320-328.

Fleishman, C.L. 1986. Genetic variation in muskoxen (Ovibus moschatus). M. Sc. Thesis. University of Alaska, Fairbanks, Alaska.

Geist, V. 1971. The relation of social evolution and dispersal in ungulates during the Pleistocene, with emphasis on the old world deer and the genus Bison. Quaternary Research 1:283-315.

Gyllenstein, U., Reuterwall, C., Ryman, N., Stahl, G. 1980. Geographical variation of transferrin allele frequencies in three deer species from Scandinavia. Hereditas 92:237-241.

Hill, W.G. 1987. DNA fingerprints applied to animal and bird populations. Nature 327:98-99.

Hatzopoulos, A.K., Schlokat, U., Gruss, P. 1988. Enhancers and other cis-acting regulatory sequences In Transcription and Splicing. Ed. Hames, B.D., Glover, D.M. IRL press, Washington, D.C.

Kocher, T.D. Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., Wilson, A.C. 1989. Dynamics of mitochondrial

evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. U.S.A. 86:6196-6200.

Lima-de-Faria, A., Arnason, U., Widegren, B., Essen-Moller, J., Isaksson, M., Olsson, E., Jaworska, H. 1984. Conservation of repetitive DNA sequences in deer species studied by southern blot transfer. J. Mol. Evol. 20:17-24.

McDonald, J.N. 1981. North American bison, their classification and evolution. University of California Press, Berkeley, Los Angeles, London.

Nei, M. 1987. Molecular evolutionary genetics. Columbia Universtiy Press, New York Guildford, Surrey.

Paabo, S., Gifford, J.A., Wilson, A.C. 1988. Mitochondrial DNA sequences from a 7000 year old brain. Nucleic Acids Research 16:9775-9787.

Paabo, S., Higuchi, R.G., Wilson, A.C. 1989. Ancient DNA and the polymerase chain reaction. J. Biol. Chem. 264:9709-9712.

Quinn, T.W., White, B.N. 1987. Identification of restriction-fragment length polymorphisms in genomic DNA of the lesser snow goose (Anser caerulescens caerulescens). Mol. Biol. Evol. 4:126-143.

Ramsey, P.R., Avise, J.C., Smith, M.H., Urbston, D.F. 1979. Biochemical variation and genetic heterogeneity in South Carolina deer populations. J. Wildl. Manage. 43:136-142.

Soldal, A.V., Staaland, H. 1979. Genetic variation in Norwegian reindeer. In: Proc. 2nd Int. Reindeer/Caribou Symposium. Pp. 396-402.

Taparowski, E.J., Gerbi, S.A. Structure of 1.711b gm/cm^3 bovine satellite DNA: evolutionary relationship to satellite I. Nucleic Acids Research 10: 5503-5515.

Thomas, R.H., Schaffner, W., Wilson, A.C., Paabo, S. 1989. Dna phylogeny of the extinct marsupial wolf. Nature 340:465-467.

White, T.J., Arnheim, N., Erlich, H.A. 1989. The polymerase chain reaction. Trends In Genetics 5:185-189.

Wilson, W. 1969. Problems in the speciation of American fossil bison In Post-Pleistocene man and his environment on the Northern Plains. Proceedings of the first annual paleo-environmental workshop. Student's press, University of Calgary, Calgary, AB.

Wilson, A.C., Cann, R.L., Carr, S.M., George, M., Gyllenstein, U.B., Helm-Bychowske, K.M., Higushi, R.G., Palumbi, S.R., Prager, E.M., Sage, R.D., Stoneking, M. 1985. Mitochondrial DNA and two perspective on evolutionary genetics. Biol J. of the Linnean Society 26:375-400.

Wilson, A.C., Ochman, H., Prager, E.M. 1987. Molecular time scale for evolution. Trends in Genetics 3:241-247.